IN VITRO STUDIES ON THE ANTIMICROBIAL PEPTIDE HUMAN BETA-DEFENSIN 9 (HBD9): SIGNALLING PATHWAYS AND PATHOGEN-RELATED RESPONSE (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

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ABSTRACT

Purpose: Human β-defensins (HBDs) are an important part of the innate immune host defense at the ocular surface. Unlike other defensins, expression of HBD9 at the ocular surface is reduced during microbial infection, but activation of toll-like receptor 2 (TLR2) in corneal epithelial cells has been shown to up-regulate HBD9. Our purpose was to test the hypothesis that TLR2 has a key role in the signalling pathway(s) involved in the overexpression or underexpression of HBD9, and accordingly, different pathogens would induce a different expression pattern of HBD9.

Methods: The in vitro RNAi silencing method and response to dexamethasone were used to determine key molecules involved in signalling pathways of HBD9 in immortalized human corneal epithelial cells. The techniques included cell culture with exposure to specific transcription factor inhibitors and bacteria, RNA extraction and cDNA synthesis, quantitative real-time polymerase chain reaction, and immunohistology.

Results: This study demonstrates that TLR2 induces HBD9 mRNA and protein expression in a time- and dose-dependent manner. Transforming growth factor-β–activated kinase 1 (TAK1) plays a central role in HBD9 induction by TLR2, and transcription factors c-JUN and activating transcription factor 2 are also involved. Dexamethasone reduces TLR2-mediated up-regulation of HBD9 mRNA and protein levels in mitogen-activated protein kinase phosphatase 1 (MKP1)-dependent and c-JUN-independent manner. HBD9 expression differs with gram-negative and gram-positive bacteria.

Conclusions: TLR2-mediated MKPs and nuclear factor-κB signalling pathways are involved in HBD9 expression. TAK-1 is a key molecule. These molecules can be potentially targeted to modulate HBD9 expression. Differential expression of HBD9 with different bacteria could be related to differences in pathogen-associated molecular patterns of these organisms.

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INTRODUCTION

Antimicrobial peptides (AMPs), also called host defense peptides, are part of the innate immune system's rapid response molecules that are expressed at epithelial surfaces of the human body. AMPs are distributed widely throughout the plant and animal kingdoms. Online databases such as http://aps.unmc.edu/AP/main.php[1] list AMPs by the hundreds in species as disparate as humans and protozoa. Their ubiquitous nature and conserved structures throughout their wide distribution indicate an important role in the evolution of host defense across the evolutionary ladder.[2] Mammalian AMPs are considered to be the eukaryotic analogues of antibiotics.[3] They are produced at the site of primary interaction of the host with the pathogen and thus serve as the first line of defense against microbial attack. They are among the early effectors of mammalian innate immunity and as such have attracted considerable attention.[4] They are also capable of signalling to host cells to trigger adaptive immune responses[5] and have many other actions that are considered below.

The ocular surface is endowed with a large variety of AMPs, where defense against environmental pathogens is paramount for preservation of sight and as a consequence on survival of the animal.[6] Activity of AMPs against culprit pathogens in ocular infections, including some drug-resistant strains, has been reported[3]; for example, human β-defensin 3 (HBD3) has been shown to be effective against *Pseudomonas aeruginosa* and *Staphylococcus* strains.[7]

HBD9 is a relatively recently discovered AMP, especially at the ocular surface, and is unique in that its expression is reduced during microbial infection. Activation of toll-like receptor 2 (TLR2) in corneal epithelial cells (CECs) has been shown to up-regulate HBD9. In this body of work, we tested the hypothesis that TLR2 plays a key role in the signalling pathway(s) involved in the overexpression or underexpression of HBD9, and accordingly, different pathogens would induce a different expression pattern of HBD9.

BACKGROUD AND LITERATURE REVIEW

Classification

Silva and colleagues[3] have classified the AMPs based on their site of synthesis into two major categories: ribosomally and nonribosomally synthesized peptides. Nonribosomal peptide synthesis is a mechanism by which the bacteria and fungi can produce bioactive metabolites used as antibiotics, antivirals, and antitumor agents.[8-10] Ribosomally synthesized peptides are an important part of the innate immune system of prokaryotic and eukaryotic organisms and contribute to their hosts' defense.[3] Marshall and Arenas (quoted by Silva et al[3]) published another classification, which divides AMPs into cationic, anionic, and aromatic peptides and peptides derived from oxygen-binding proteins. Of these, cationic peptides are widely distributed in the animal and the plant kingdoms, and thus they are the largest group of AMPs and the first reported.[11]

There are three main groups of AMPs found in humans based on the different motifs between groups, namely, defensins, cathelicidin, and histatins.[12] Defensins are small, 12-50 amino acids, highly cationic, cysteine-rich AMPs with a net positive charge

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due to an excess of basic lysine and arginine residues with disulphide bridges[13] determining their secondary folding.[4,5] They share certain common elements of structure, a tendency to synergism and a host of actions from direct microbicidal to cell signalling, and control of inflammation, and even possess anticarcinogenic and antiangiogenic properties.[5,14,15] The latter may be pertinent to the therapy of age-related macular degeneration. Of the three groups, defensins are the most researched. These can be further divided on the basis of their structure into α-defensins, β-defensins, and θ-defensins.

Six α-defensins and five β-defensins have been identified in humans. Defensins 1 through 4 are found in high concentrations in the azurophilic granules of leukocytes, though human neutrophil α-defensin 4 is approximately 100-fold less abundant.[16-18] They are also expressed by monocytes and lymphocytes.[17] Human α-defensins 5 and 6 are a feature of the prominent apical granules of the Paneth cells of the small intestine.[19-21] The former has been isolated from the female reproductive tract as well.[22] β-Defensins are produced by a variety of primarily epithelial cells. Their expression is both constitutive and induced. Many animal and cell culture models, as well as studies on human impression cytology specimens, have reported the constitutive or inducible nature of some of these myriad peptides in response to different stimulants, the more well known being the α-defensins 1 through 4 and the human β-defensins 1 through 4 and 9, and leucine, leucine-37 (LL-37).[23-27] Other, less researched AMPs include liver-expressed AMPs (LEAP-1 and -2) and many that still remain putative.[15,28,29] This last fact and the promise that these moieties hold as therapeutic agents underscore the importance of this family of peptides.

The θ-defensins or retrocyclins are octadecapeptides that are expressed in the bone marrow and leukocytes of rhesus macaques, but because of a premature stop codon in the *Homo sapiens* gene and messenger ribonucleic acid (mRNA), their expression has been silenced in human leukocytes.[30] The structure of θ-defensins is different from that of α-defensins and β-defensins.[13] The N-termini of θ-defensins are covalently linked to their C-termini through peptide atoms, and thus they are the only cyclized defensins. Six θ-defensins have been identified: retrocyclins 1 through 3 and rhesus θ-defensin 1 (RTD1), RTD2, and RTD3.[5]

The AMPs histatins are also small histidine-rich, cationic peptides found in human saliva and have antimicrobial and antifungal actions. They assume a random coil conformation when in solution in water.[31] Cathelicidins are 12 to 80 amino acid peptides derived from the human 18 kDa cationic antimicrobial protein (CAP18) following proteolytic digestion of the C-terminal end. Cathelicidins are largely found in the lysosomes of neutrophils and macrophages.[32]

Cationic peptides also can be classified on the basis of their structural characteristics into three main classes: linear peptides forming α-helix structures such as cathelicidin; cysteine-rich open peptides containing one or more disulphide bonds, such as defensins; and molecules rich in specific amino acids, such as histidine in histatins.[3,11,33]

Actions

AMPs have both microbicidal and nonmicrobicidal actions. The latter include such diverse actions as immune activation and suppression, wound repair, angiogenesis, adrenocorticotrophin hormone activities, anticancer properties, and contraception.[5,14,15]

AMPs were first known for their direct ability to kill microbes, gram-positive and gram-negative bacteria, fungi, eukaryotic parasites, and viruses.[34-36] Regarding the latter, they are known to be active primarily against enveloped viruses[37,38] but do also act against nonenveloped viruses, such as adenovirus species[15,36,39] and papillomavirus.[40] The defense system of *Drosophila* has been demonstrated to involve a large family of AMPs with a host of functions against diverse classes of organisms,[41] and this concept is likely to be valid for humans too. AMP antimicrobial activity has been deduced by work such as that done by Wilson and associates[42] wherein the gene for the tissue metalloproteinase matrilysin, which is responsible for posttranslational proteolytic cleavage of Paneth cell α-defensins, was disrupted in mice and these succumbed more readily to exogenous *Escherichia coli* and *Salmonella typhimurium* bacteria. α-Defensins and β-defensins have reduced activity at physiological salt concentrations[43] so that their optimal actions probably occur within the phagocytic vacuoles of phagocytes and on the mucosal and skin surfaces, where there is a low ionic milieu. A study by Huang and coworkers[7] shows the importance of HBD3 in the antimicrobial action on the ocular surface, as its activity was maintained in the presence of human tears.

Another important facet of AMP action is synergy between a number of peptides, such that a shortfall in amount is balanced by an excess in numbers[44]; LL-37 and HBD2 act synergistically against *Saureus*[45] and the activity of HBD3 is boosted by lysozyme.[46] It is not only the killing properties of AMPs that are enhanced by the synergy but also the expression of certain cytokines; for example, Niyonsaba and coworkers[47] have demonstrated the production of the chemokine IL-8 by the concerted action of HBD1 through 4 and LL-37. AMPs even synergize with antibiotics.[44,48,49] However, unlike antibiotics, which do not act against fungi and viruses, AMPs are able to act across the microbial spectrum. Their antimicrobial action is as good against normal bacteria as against multidrug-resistant *P aeruginosa* or methicillin-resistant *S aureus*.[50,51]

A detailed account of the mechanisms of microbial killing can be found in an excellent review by Yeaman and Yount.[52] In brief, initial host-microbe membrane interaction by means of electrostatic attraction or receptor interaction is followed by conformational change, concentration, and multimerization of AMPs. Actual processes of killing may be any of the following: (a) Barrel-stave mechanism, wherein peptides align in a barrel-like ring around a membrane pore with some stavelike peptides crossing the pore. Increasing peptide concentration increases the pore size and allows them to insert deeper to gradually lyse the membrane. (b) Toroidal pore/wormhole mechanism, in which peptides intercalated with lipids form a transmembrane channel. Displacement of membrane components destabilizes the membrane, dissembling the pore and allowing more peptide to access the inner cytoplasmic leaflet and therefrom reach potential intracytoplasmic targets. (c) Carpet mechanism, wherein peptides act diffusely like detergents to lyse the microbial membrane without intramembrane insertion but by dense accumulation at the surface, affecting the membrane phospholipid integrity. Actual mechanisms of cell death include not only membrane dysfunction but inhibition of extracellular biopolymer synthesis and inhibition of intracellular functions, "a multi-hit process."[52]

Ocular Profile of AMPs

As with other organ systems, AMPs are abundantly found in ocular tissue. Stolzenberg and colleagues[53] discovered lingual antimicrobial peptide (LAP, so called as it was first found in the tongue) in bovine conjunctival epithelium in 1997. We first reported α-defensin in normal and diseased human corneas in 1998[54,55] followed by our observations[56] on the presence of β-defensins 1 and 2 in human cornea and conjunctiva but only the former in lacrimal gland tissue. We and others then confirmed the presence of HBD1 mRNA in the lacrimal gland, aqueous, iris, ciliary body, lens epithelium, and vitreous.[56-58] We found α-defensins 1 through 3 to be present in lacrimal tissue, tears, and inflamed conjunctiva, but α-defensins 5 and 6 were not detected. A great deal of work has been done studying the protein profile of tears[59-61] showing that the α-defensins 1 through 3 are consistently present and related to inflammation.[62] It is presumed that these defensins originate from neutrophils present in tears.[56] β-Defensins appear to be absent from the tear film.[62] Our study in 2005[6] has described the constitutive expression of HBD1 and 2 in all human ocular samples studied, which included CEC cultures from limbal explants, conjunctival and CECs obtained by impression cytology, and CEC obtained by mechanical removal from cadaver eyes.

Several other studies[54,58,63] have supported the constitutive and induced expression of both HBD1 and 2. However, McDermott's group was able to demonstrate constitutive expression of HBD2 in only two of eight cadaveric corneal epithelial samples in one study,[64] and in another[65] only in dry eye conjunctival epithelial samples as opposed to normal controls. Corneal and conjunctival epithelia express HBD1 and HBD3 constitutively, whereas HBD2 is variably expressed in normal tissue only occasionally.[16,17,19-22] HBD2 is expressed more in ocular surface inflammation and infection, because lipopolysaccharides and proinflammatory cytokines that are produced by the bacteria up-regulate the expression of HBD2.[66]

While we demonstrated constitutive expression of HBD2 in ocular surface epithelium,[57] we were unable to detect constitutive expression of HBD2 in human postmortem ciliary body or in aqueous and vitreous samples.[57] Others confirmed the constitutive expression of HBD1 mRNA in the lacrimal gland, aqueous, iris, ciliary body, lens epithelium, and vitreous but could not demonstrate HBD2 in any of the intraocular samples.[56-58] There is thus no strong consensus on the constitutive nature of HBD2 expression.[67] HBD2 expression is known to be inducible[68,69] and is up-regulated in conditions of re-epithelialization of the cornea[70] and also in the severe dry eye condition of Sjögren syndrome.[65,71] The specific role of AMPs inside the eye still needs further investigation. Owing to the broader-spectrum effect and higher potency of HBD2, it can be stronger in antimicrobial activity inside the eye than HBD1.[57] HBD3 has been classed as a constitutive AMP of the human conjunctival and corneal epithelium.[65] Our earlier work[6] did find presence of this defensin in impression cytology samples of cornea more than in conjunctiva and in all cultured corneal epithelial samples. Further recent work by us on impression cytology samples from normal and diseased states revealed constitutive expression of HBD3, but it was highly expressed in a significantly larger number of *infected* corneal and conjunctival samples, showing an inducible nature.[72] This is supported by other studies that have shown HBD3 up-regulation after treatment with tumor necrosis factor (TNF) α and interferon-γ.[73] Narayanan and associates[65] did not find an up-regulation of HBD3 after 24-hour treatment with either interleukin (IL)-1β, IL-β and TNF-α, or heat-killed *P aeruginosa*. One possible explanation could be the source of cells used, as this was not consistent across the studies reported. Cell lines, cultured cells, cadaveric corneal scrapes, and impression cytology samples were all employed in the AMP studies. With impression cytology it is possible to obtain only the superficial two or three layers of cells, and it is possible that there is a differential expression of AMPs between basal, suprabasal, and superficial cells. We also evaluated HBD4 expression on the ocular surface and found it in only a single conjunctival epithelial impression cytology sample and two corneal samples, but in *all* cell culture samples.[6] This indicates that the highly stimulatory growth medium or postmortem changes or even the harvesting of deeper cell layers from cell culture samples, as opposed to only superficial layers by impression cytology, may be relevant to HBD4 expression. Further work is needed to elucidate this. Later, McDermott[74] referred to her own unpublished work and stated that they were not able to provide any evidence of HBD4, 5, or 6 expression in either corneal or conjunctival epithelial cells. Other studies looked at the expression of HBD4 and found that it is common in cultured ocular surface cells, but only infrequently in actual tissue samples.[6,7,75] Of relevance to the work presented in this study, we have reported that HBD9 is expressed by corneal and conjunctival epithelial cells.[76,77]

Psoriasin, another AMP, was found to be expressed constitutively in corneal and conjunctival epithelium and up-regulated in response to some bacterial products.[78] We also reported the corneal and conjunctival expression of ribonuclease-7 (RNase-7) with the up-regulation in response to IL-1b.[79] Various animal cell culture models of defensin production have been used.[80,81] Bovine conjunctival epithelium has been used to illustrate tracheal antimicrobial peptide production (TAP, HBD-2 homologue) and its up-regulation in injury.[81] We[6] have investigated a number of putative AMPs, DEFB (defensin-β) 105, 107, 108, 118 through 123, 125 through 127, and 129, proposed to be present in the human genome on the basis of a hidden Markov model (a statistical model in which the challenge is to predict the hidden parameters from the observable parameters) and also present in expressed sequence tag (EST) libraries (short subsequences of transcribed genes with or without protein expression [http://www.ncbi.nlm.nih.gov/guide/all/[82]]). These were all absent from ocular surface cells.

LL-37, the human cathelicidin, has been detected by mass spectrometry in the tear film[83] and shown to be responsible for strong microbicidal activity as well as corneal epithelial migration.[84] We were also able to detect it in a majority of samples.[6] Azurocidin of neutrophils[85] has also been found to be expressed by rabbit corneal epithelium, stromal fibroblasts, and bulbar conjunctiva when challenged with *Staphylococcus aureus* and by human CEC lines in the presence of IL-1β and TNF-α.[86] Azurocidin may act to alarm the immune system, acting as a mediator in the initiation of the immune response and as a chemo-attractant and activator of monocytes and macrophages. It functions through enhancing the cytokine release and bacterial phagocytosis to allow for more bacterial clearance.[28] Thymosin-β4 is an *anionic* antimicrobial peptide of platelets with antimicrobial,[87] among other, properties[88] and appears to promote corneal epithelial migration and cytokine production in an alcohol injury model.[89] Similar wound healing but with

a lowered cytokine milieu is seen in cases of alkali injury.[90]

Our work[6] has also revealed mRNA for the LEAP-1 (also known as hepcidin)[66] and LEAP-2, in a large number of ocular surface cell samples and dermicidin in only one corneal sample. However, this may have come from cells of the eyelid skin, as this AMP is a constitutive component of the eccrine sweat glands.[91,92] The chemokine CCL20 (C-C motif ligand 20), a known antimicrobial[93] inducible by IL-1α, has ocular gene expression.[94] CCL28 is another ocular chemokine with antimicrobial activity.[74]

In summary, the antimicrobial milieu of the ocular surface is composed of the β-defensins 1 through 4 and 9, LL-37, LEAP-1 and 2, and aqueous secretions of the lacrimal gland supported by a low-level presence of polymorphonuclear neutrophil–derived α-defensins and azurocidin. The aqueous tear film contains lactoferrin, lysozyme, and lipocalin-1 in addition to other antimicrobial moieties, such as secretory immunoglobulin A (sIgA, a dimer that is present at greater concentrations in the morning than in the afternoon[95]), immunoglobulin G (IgG), complement components, β-lysins, orosomucoid, and caeruloplasmin, all of which prevent bacterial adherence and kill organisms.[96, 97] This is complemented by the secretions of the apocrine glands of Moll near the eyelashes, which also contain lysozyme, HBD-2, adrenomedullin, lactoferrin, and IgA.[98]

Mucin of the tear film is another important player that thwarts bacterial adherence to the cornea.[63] Inhibition of bacterial growth by ocular mucins can be seen as an element in the system of mucosal control of microbiota.[99] Bactericidal/permeability-increasing protein (BPI) is in high concentration in human tears and appears to have an important antibacterial role.[100,101] Synergy exists between lysozyme and lactoferrin,[102,103] and these in turn synergize with HBD2.[104] It is clear that synergy between peptides is a major feature of their activity.[105-107] As already stated, this appears to be a means of keeping the concentrations of the individual peptides low and thus circumvent any deleterious effects on host cells; LL-37 can be toxic to lymphocytes and other leukocytes,[108] as can the α-defensins.[109]

Other tear components with antimicrobial properties do exist with broad-spectrum activity; in fact, the antimicrobial effect might not be their primary function.[59] Examples include the enzyme secretory phospholipase A_2,[110,111] the secretory leukocyte protease inhibitor,[112,113] elafin,[114] surfactant protein A and D,[115-117] psoriasin,[78] dermcidin,[118] histatin,[59] and lacritin.[119]

In light of these findings, some reports on the negative effects of lacrimal secretions on antimicrobial activity have been confounding.[120] These effects occur through a high salt content, known to inactivate these peptides,[43,121] and also by means of serine protease inhibitors, which react with and inactivate defensins.[122] However, this may simply imply a means of keeping potential AMP-induced cytotoxicity in check at an important mucosal surface, damage to which may have serious implications in terms of scarring and opacification of the cornea or even sight-threatening neovascularization. This underscores the importance of a tight control of AMP secretion that is required for host defense, as any overexpression may have a deleterious effect. The nasolacrimal duct epithelium, too, as part of the ocular mucosal immune system, expresses a number of AMPs.[123,124] Additional protection is afforded by the antimicrobial constituent-rich tear film that bathes the nasolacrimal passages.

It is not clear which nonmicrobicidal actions by which specific peptides and in which synergistic combinations occur on and in the eye. We know that the innate immune system as manifested at the ocular surface, with its antimicrobial substances, exchanges signals with the adaptive immune system.[63,125] These can only be postulated by extrapolation from the many mechanisms described above on other tissues. However, there are some pointers, one being that α-defensins seem able to stimulate conjunctival goblet cells to secrete mucins[126] in the same way that they stimulate lung epithelial cells.[127] These findings provide strong evidence of a major role of these AMPs in immune and allied processes on the ocular surface, but how these translate to actual in vivo activity needs further study.

New AMPS at the Ocular Surface

As briefly mentioned above, we have discovered a new AMP of the β-defensin family, DEFB-109, at the ocular surface by means of both conventional and real-time polymerase chain reaction (PCR) and have been intrigued by its down-regulation in disease processes.[76] In another recent in vitro study with acanthamoeba, we demonstrated that HBD9 expression is initially down-regulated and then increases.[72] The RNase-A superfamily has gained attention because of its newly identified role in host defense. Of these, RNase-7 and its orthologous gene RNase-8 have been described as possessing antimicrobial activity against a variety of pathogens.[128] We have demonstrated the constitutive mRNA expression of RNase-7 in both in vivo impression cytology specimens and ex vivo CEC cultures. Increased regulation of RNase-7 transcript was noted in diseased patient samples and CEC cultures incubated with inflammatory cytokines (IL-1β and TNF-α).[79]

This indicates that there are probably many more AMPs to be discovered in relation to the eye and raises the hope that their characterization may ultimately yield a valuable therapeutic resource and a better understanding of the interactions between innate and adaptive immune responses.

AMPs and Disease

Knowing as we do the important role of AMPs in preventing infection, it is not surprising to note altered AMP expression in disease states. There are a number of diseases in the nonocular context related directly to abnormal expression of AMPs. A severe recessive disorder by the name of morbus Kostmann, or infantile genetic agranulocytosis,[129] occurs as a result of lack of the cathelicidin LL-37 and α-defensins HNP 1 through 3 in neutrophils. In addition, LL-37 has been found to be totally absent from plasma and saliva of affected individuals. Patients suffer from recurrent periodontitis and chronic gingivitis and require recombinant granulocyte-monocyte colony-stimulating factor to replace neutrophils.[130] Specific granule deficiency is another disorder in which neutrophils are lacking in defensins[131] and other proteins.[132] Chronic pulmonary infections in cystic fibrosis are believed to occur because of functional inhibition of β-defensins by hypertonic airway surface fluid.[43] Patients with atopic dermatitis have decreased levels of LL-37 and HBD-2 and are susceptible to infections as a consequence.[133] On the other hand, increased levels of AMP expression occur in psoriasis[134,135] and protect against superadded infection.[136] Active ulcerative colitis and Crohn disease are related to high levels of neutrophil defensins and lysozyme, which are not seen in normal mucosa.[20]

There are limited studies that correlate AMPs expression and eye disease. We have demonstrated the down-regulation of the otherwise constitutively expressed novel AMP, DEFB-109, in all of the disease states of the ocular surface studied, ie, bacterial keratoconjunctivitis, viral keratitis, acanthamoeba keratitis, and dry eye disease.[76] This has implications for understanding the role of this and other AMPs of the defensin family, in pathological conditions of the ocular surface. It is likely that some nonmicrobicidal functions are also affected at this (and possibly other) mucosal surfaces and may be amenable to modulation to manage disease. We have found HBD1, 2, and 3, LL-37, and LEAP-1 and 2 to be the AMPs commonly expressed at the ocular surface.[6] In our study of these AMPs, in the conditions named above, using real-time PCR on impression cytology samples from patients, we showed that expression of specific AMPs was related to the nature of infecting organism, for example, viral keratitis specifically up-regulated LEAP-1 and the TLRs 8 and 10 and bacterial keratitis up-regulated HBD2 and 3.[6]

Non-Sjögren dry eye exhibits up-regulation of HBD2 with resultant T-cell chemotaxis and histamine release from conjunctival mast cells causing the ocular irritation of dry eye.[65,137] Brito and colleagues[138] were the first to report coexpression of the two endotoxin receptor proteins, cluster differentiation 14 (CD14) and TLR4, intraocularly, ie, in ciliary body, explaining the sensitivity of the iris/ciliary body to endotoxin. They showed up-regulation of TNF-α, IL-1β, IL-6, and IL-8 by endotoxin treatment and proposed that these receptors were responsible for the acute sensitivity of the uvea to endotoxin-induced uveitis. It remains to be seen if there is a role for AMPs in this setting, as they are closely related to TLRs. These studies indicate that certain AMPs are closely related to the disease conditions studied, and their differential expression may be either the cause or the effect of the disease process. This is another area requiring further clinical research.

Therapeutic Implications of AMPs and the Future

The therapeutic potential of AMPs has driven research in this field. Over 600 AMPs have been isolated from a large variety of organisms.[3] The idea of developing AMPs as drugs is an intriguing one, and the race to do so is gaining momentum. Natural and synthetic antimicrobial peptides have been shown to be effective in reducing microbial titers in a number of preclinical studies.[35,139-142] Gramicidin S and polymyxin B are two topical AMPs already in use.[143]

In terms of ocular therapy, the progress is slow. Xoma is a company that may put one of its products, BPI, to use as an anti-infective agent for keratitis.[74] Contact lens cleaning solutions containing AMPs may be more powerfully antimicrobial than conventional agents,[144] and corneal storage media may benefit likewise from the antimicrobial and preservative action of certain AMPs.[145] Promising in vitro anti-infective activity of a number of AMPs has, unfortunately, not translated to in vivo efficacy, and more needs be done to manipulate them toward clinical relevance.[146] Any clinical antimicrobial activity will have to be considered in the context of possible adverse nonmicrobicidal effects of these agents at the dosage applied. Much more work is required in this context before both safety and efficacy can be balanced for therapeutic application.

INTRODUCTION TO PROJECT

Human β-defensins are cationic AMPs that have antimicrobial properties and also other roles in relation to the immune system. They act as effectors of the innate immune system. On the basis of genomic information, it can be predicted that the human genome can encode for tens of β-defensins.[147] The first human β-defensin, HBD1, was discovered in 1995.[148] Human β-defensins are expressed at a variety of mucosal surfaces, including the ocular surface. HBD1 and HBD2 are constitutively expressed, whereas HBD3 is expressed only in response to infection or inflammation.[6,149] Several studies have elucidated the relationship between TLRs and the production of β-defensins. Vora and colleagues[150] have reported the role of TLR2 and TLR4 in up-regulation of HBD2 expression in intestinal epithelium via activation of kappa-light-chain-enhancer of activated B cells (NF-κB) or activating protein 1 (AP-1) transcription factor. Likewise, keratinocytes stimulated with *Propionibacterium acnes* produced HBD2 in TLR2- and TLR4-dependent fashion.[151] Involvement of TLR2 activated NF-κB, JNK, and p38 signalling pathways in induction of HBD2 treated with *S aureus* and (palmitoyloxy)$_3$-cysteinyl-serine-(lysine)$_4$ (Pam3CSK4) has also been demonstrated in corneal epithelium.[149]

Human β-defensin 9 (HBD9) is a relatively recently described member of the defensin family, which was found to be down-regulated in *Candida albicans*–treated gingival epithelial cells.[152] This reduced expression was unique to HBD9 compared to β-defensins HBD2 and 3, expression of which is increased in response to bacterial infection. The investigators attributed this to an escape strategy by the host that encourages commensal flora to flourish.[14] Our group was the first to demonstrate the presence of HBD9 (gene DEFB109) at the ocular surface.[76] We also demonstrated low levels of HBD9 mRNA in impression cytology samples of the ocular surface taken from healthy subjects and patients with infection or inflammation.[77] In our in vitro study using immortalized human corneal epithelial cells (HCECs),[77] we demonstrated that when HCECs were exposed to pathogen-associated molecular patterns (PAMPs) that interact with receptors of the innate immune system, such as TLRs, nucleotide oligomerization domain-like receptors (NLRs), and interleukin-1 receptor (IL-1R), a rapid induction of HBD9 mRNA occurred. Of all receptors, activation of TLR2 induced the maximum expression of HBD9 mRNA.

Building on our continuing work on AMPs at the ocular surface,[6,54,56,57,72,76,77,79,153,154] and specifically on our recent work on the characterization of the signalling pathway involved in the expression of RNase-7, another potent antimicrobial peptide of the skin and ocular surface,[79] we hypothesized that TLR2 would be key to the signalling pathway(s) involved in the expression, up-regulation, or down-regulation, of HBD9 and that different pathogens would induce a different expression pattern of HBD9.

The aim of this study was to identify biomarker-signalling molecule(s) (with potential for therapeutic exploitation) involved in the induction of HBD9. We used in vitro RNA interference (RNAi) silencing method and exposure to dexamethasone as a means to characterize the signalling pathways involved in HBD9 expression in response to activation of TLR-2 in HCECs. In addition, we examined the expression of HBD9 mRNA in comparison to other AMPs, following infection with a gram-negative bacterium (*P*

aeruginosa) and a gram-positive bacterium (*S aureus).*

METHODS

The research was conducted in accordance with the tenets of the Declaration of Helsinki. Local ethics committee and the Research and Development Department of the National Health Service Trust approved this research (reference numbers: OY100201 and Q1110207).

CELL CULTURE

Simian virus (SV) 40 immortalized transformed HCECs were kindly gifted by Dr F. Rose (School of Pharmacy, The University of Nottingham, United Kingdom). This cell line was established by Araki-Sasaki and coworkers[155] by infecting primary human cultured cell lines with recombinant SV40 adenovirus vector. The cells exhibit cobblestone morphology and demonstrate desmosomes and microvilli in culture and stratify when subjected to an air-fluid interface. This cell line model remains stable until 400 generations and is currently being widely used for studying the innate immune signalling pathways on corneal epithelium. Passages 4 through 18 (spread over14 generations of HCECs) were used in this study.

Cells were maintained in cell culture medium (Epilife; Cascade Biologics, Paisley, United Kingdom) containing human keratinocyte growth supplement (HKGS; Cascade Biologics), antibiotic mixture (gentamicin and amphotericin B; Cascade Biologics), and antimycoplasma agent (Plasmocin; InvivoGen, Europe).

The HCECs were grown in T75 flasks containing EpiLife medium, maintained in an incubator (Sanyo Electric Co Ltd) in humidified conditions with 5% CO_2 at 37˚C, and maintained until 70% to 90% confluence was attained. This was ascertained by phase contrast microscopy (Nikon Eclipse TS100, Japan). Before treatment with pathogens or stimulants, HCECs were starved overnight in HKGS-free media.

TREATMENT OF HCECS

Human β-Defensin 9 Studies

Cells were pretreated for 30 minutes without (control, diluent + serum-free EpiLife) or with three specific mitogen-activated protein kinase (MAPK) pathway inhibitors (SB203580, SP600125, and PD98059, respectively) or NF-κB pathway inhibitors (NF-κB activation inhibitors and SC514) at concentrations specified in the respective Figure legends. Cells were then washed with 1 to 3 mL phosphate-buffered saline (PBS) solution using a 1-mL Pasteur pipette. This treatment ensured that no residual inhibitor solution was left in the wells. The PBS was discarded and cells were then incubated in absence or presence of desired ligand for specified duration at 37˚C. Following treatment, supernatant and cell-lysate (for total RNA extraction) were collected individually and stored at -80°C until further analysis.

Bacterial Exposure

P aeruginosa as a representative of gram-negative bacteria and *S aureus* as a representative of gram-positive bacteria derived from pathogenic human ocular isolates were used in this study (obtained from the Microbiology department of the Queens Medical Centre, Nottingham, United Kingdom). The bacteria were cultured in a blood agar medium prior to the stimulation studies and were then stored in a 1:1 mixture of brain-heart infusion broth (BHIB) and glycerol at -80°C. Prior to experimentation, bacteria were inoculated onto blood agar plates and incubated overnight at 37°C with 5% CO_2. Thereafter they were expanded by growing in BHIB medium in the orbital shaker overnight at 37°C.

After 24 hours, 5 mL of the culture was centrifuged at 112*g* for 15 minutes, and after washing with PBS, the pellet was resuspended in 10 mL of EpiLife by vigorous vortex mixing. The optical density of the bacterial suspension was measured at 620 nm and normalized to 0.2 by dilution in EpiLife. The cytotoxic/pathogenic effects of serial dilutions of the bacterial suspension on HCECs were assessed microscopically. With both bacterial isolates, dilutions of 1:8 or higher were found to have no observable effects on cell morphology after 24 hours of incubation. Henceforth, a 1:10 dilution of the normalized suspension was used to challenge HCECs. The colony-forming units (CFUs) of each test suspension were determined by plate count and found to be 1.3×10^7 and 2.1×10^7 CFU for *S aureus* and *P aeruginosa*, respectively. Subsequently, confluent HCECs were incubated with 1.5 mL of *P aeruginosa* or *S aureus* suspensions, and samples of HCECs were collected at 1, 3, 6, and 9 hours. Control samples were obtained from nonstimulated HCECs. At each time point, the culture media was removed and 700 µL of buffer RLT (Qiagen) was added to each well for 15 minutes until the cells were fully solubilized, and the lysate was collected and stored at -80^o C. Also, the supernatant suspensions of *P aeruginosa* and *S aureus* (without HCEC cells) were collected separately and centrifuged for 2 minutes at 287*g*. Finally, 700 µL of buffer RLT was added and the samples were kept at -80°C for further analysis. All tests were done in triplicate with cells at the same passage number.

ISOLATION OF RNA AND CDNA SYNTHESIS

Total RNA was extracted from each sample using RNeasy Mini Kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. Briefly, the HCECs were treated with Buffer RLT to lyse the cell and nuclear membranes, allowing for complete release of total RNA from the sample. The cell lysate was poured onto Qiashredder column and spun for 2 minutes at 12,000 rpm. The filtrate was collected and equal volume of 70% v/v ethanol was added, providing specific conditions that promote selective binding of RNA to the RNeasy silica membrane. The sample was applied to the RNeasy mini spin column where RNA binds to the membrane, and sample contaminants were effectively washed away during Buffer RW1 and Buffer RPE (patent buffers, Qiagen)

washing stages. In the final step, RNA was eluted in 13 µL RNase-free water. The use of RNase-free water is essential as even minute amounts of RNases can affect the synthesis of complementary DNA (cDNA) and sensitivity in quantitative PCR (qPCR). All the binding, washing, and elution steps mentioned above were performed with stepwise centrifugation using a microcentrifuge (MicroMax, Model 230, International Equipment Company [IEC]). Total RNA was eluted in RNase-free water (Qiagen) and quantified (NanoDrop Spectrophotometer; Thermo Fisher Scientific, Loughborough, United Kingdom).

Reverse Transcription of Total RNA

Using the QuantiTect RT kit (Qiagen), 2000 ng of total RNA was reverse transcribed to cDNA. Calculations were performed to establish the amount of purified RNA required per sample for cDNA synthesis; for example, one sample measured 813.5 ng/µL. This was divided by 2000, which meant 0.41 µL of the sample was required in this instance. The purified RNA samples were then heated with genomic DNA Wipeout Buffer (gDNA WB; provided in the kit) to 42°C on a thermal cycler (Hybaid Ltd, United Kingdom) for 2 minutes to eliminate contaminating gDNA. The samples were then reverse transcribed using a mastermix containing QuantiTect RT enzyme, QuantiTect RT Buffer, and RT Primer Mix. Samples were heated to 42°C for 30 minutes, followed by deactivation of the RT enzyme by heating samples to 95°C for 3 minutes. These were then stored at -20°C ready for qPCR. Eluted RNA not used for cDNA synthesis was re-stored at -80°C. For each batch of samples, one sample was made up, which did not contain RT, acting as negative control. Although current practice (RNeasy Mini Kit protocol; Qiagen) was very reliable and known to remove the vast majority of cellular DNA, it does not guarantee complete removal. For this reason, samples without RT enable detection of any residual DNA in the material when analyzed using PCR.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

qPCR was performed to measure the relative abundance of gene of interest (GOI) mRNA. Custom-made Taqman assays (Applied Biosystems, United Kingdom) were used for endogenous control 18s rRNA, hypoxanthineguanine phosphoribosyltransferase (HPRT), HBD 1 through 3 and 9, LEAP1 and 2, LL37, and RNase 7. qPCR reactions were run using 8-strip optical tubes (Stratagene, Europe) in the Mx3005p real-time PCR system (Stratagene). qPCR setup was carried out in an aseptic manner and using nuclease-free containers, solutions, microfuge tubes, and pipette tips. All samples and chemicals were kept on prefrozen trays to ensure no degradation occurred. qPCR experimental setup was performed as detailed in the manufacturer's protocol (Applied Biosystems). Initially, template cDNA (prepared from 2000 ng of total RNA) of desired samples was diluted to 1:5 using nuclease-free water to perform the qPCR in triplicate. Each reaction was prepared to 20 µL final reaction volume with 10 µL of 10x Taqman gene expression master mix, 1 µL of Taqman assay (contains primer and probe mix), 5 µL of diluted cDNA, and 4 µL of nuclease-free water. Each 96-well plate was run with the GOI and respective endogenous control Taqman assay in triplicate. Appropriate negative controls (nontemplate control and reverse-transcriptase control) and positive control (Human Reference RNA; Stratagene) were also run. Details of the target genes and primer codes used in the study are listed in Table 1.

DATA ACQUISITION AND ANALYSIS

Data were gathered using the MxPro software (version 4.01; Stratagene) on the computer linked to the qPCR machine. The raw data was then transferred to Microsoft Excel spreadsheet and analyzed using the $\Delta\Delta C_T$ (delta-delta threshold cycle) method.[156] First, the ΔC_T value for each sample was determined by calculating the difference between the C_T value of the target gene and the C_T value of the endogenous reference gene in that sample. This was determined for both the control (calibrator) and all disease samples.

$\Delta C_{T(sample)} = C_{T\ G.O.I} - C_{T\ HPRT/18s}$

$\Delta C_{T(control)} = C_{T\ G.O.I} - C_{T\ HPRT/18s}$

Therefore,

$\Delta\Delta C_{T} = \Delta C_{T(sample)} - \Delta C_{T(control)}$

A greater normalized C_T value indicated a higher level of expression. Since the control group was used as the baseline with which to compare down-regulation or up-regulation of a gene in the various groups studied, an average for the $2^{-\Delta\Delta CT}$ value of samples in the control group was calculated and then the individual value of each control sample, as well as each study group sample, was divided by that average value to get as clear as possible differences between the control and study groups. Standard deviation (SD) and standard error of the mean (SEM) were calculated for each group by standard formulas in Excel (Microsoft Office). This allowed graphical representation of data with indicators of significance in differences from the control samples.

Statistical Analysis

The qPCR data was statistically analyzed using SPSS 16.0v software. The statistical significance was set at $P \leq .05$. For the pathogen challenge study the Student *t* test was used to statistically compare the gene expression of the AMPs studied in challenged samples with those obtained from unchallenged controls. All data were represented as means and standard errors (SEs) of two independent experiments performed in triplicate.

CELL IMMUNOFLUORESCENCE AND MICROSCOPY

Immunfluorescence staining was performed using established techniques. The HCECs (2×10^4) seeded onto 4-chamber slides (Lab-Tek, United Kingdom) and treated with various reagents were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. Samples were then blocked with normal goat serum (1:10) for 30 minutes followed by incubation with polyclonal primary antibody against HBD-9 (rabbit anti-human; 1:50 dilution, Eurogentec, United Kingdom) overnight at 4°C. Samples were washed and incubated with secondary antibody (goat anti-rabbit IgG, 1:300 dilution; Invitrogen, United Kingdom) for 1 hour, to

detect primary antibody. The counterstaining was performed with 4′, 6-diamidino-2-phenylindole (DAPI; 1:250) and examined with an Olympus BX51 fluorescent microscope (Olympus, United Kingdom) at ×400 magnification and photographed. The panels display merged images of both HBD9 (yellow) and nuclei (DAPI; blue). Data shown are representative of three independent experiments.

TABLE 1. DETAILS OF THE HUMAN GENES AND PRIMER CODES USED TO AMPLIFY DEOXYRIBONUCLEIC ACID OF THE DIFFERENT ANTIMICROBIAL PEPTIDES STUDIED.

GENE SYMBOL	TAQMAN ASSAY ID	ACCESSION NUMBER
HPRT	4333768F	NM_000194.1
DEFB1/Hs00174765_m1	Defensin, beta1/HBD1	NM_005218.3
DEFB4/Hs00823638_m1	Defensin,beta4/HBD2	NM_004942.2
DEFB103A/Hs00218678_m1	Defensin,beta103A/HBD3	NM_018661.2
CAMP/Hs00189038_m1	Cathelicidin AMP	NM_004345.3
HAMP/Hs00221783_m1	Hepcidin AMP (LEAP 1)	NM_021175.2
LEAP2/Hs00364834_m1	Liver-expressed AMP2	NM_052971.2
Hs00261482_m1	RNase7	NM_032572.3
Hs02760065_g1	DEF109	NM_001037380

RESULTS

TOLL-LIKE RECEPTOR 2 (TLR2) MODULATES HBD9 MRNA EXPRESSION IN HCECS

Toll-like receptors are a family of molecules that play a key role in innate immunity by facilitating pathogen recognition in relation to PAMPs expressed on invading pathogens. Different TLRs demonstrate different patterns of expression. TLR2 is one member of the TLR family. In a previous study we showed that HCEC demonstrated rapid up-regulation of HBD9 in response to Pam3CSK4.[77] Pam3CSK4 is a synthetic lipopeptide (LP) that is very similar to the active terminus of bacterial LPs. It stimulates the proinflammatory transcription factor NF-κB via TLR2. To further understand the role of TLR2 in HBD9 expression, we stimulated HCECs with different concentrations of Pam3CSK4 (Invogen, United Kingdom) (50 to 1000 ng/mL) for 1 hour. As shown in Figure 1, we noted a dose-dependent increase in HBD9 mRNA expression in response to TLR2 activation. With 50 ng/mL Pam3CSK4, a 2.83-fold increase in HBD9 mRNA (*P*=.0010) was observed. Notably, with 1000 ng/mL, HBD9 mRNA increased to 5.32-fold (*P*=.0001) compared to untreated control.

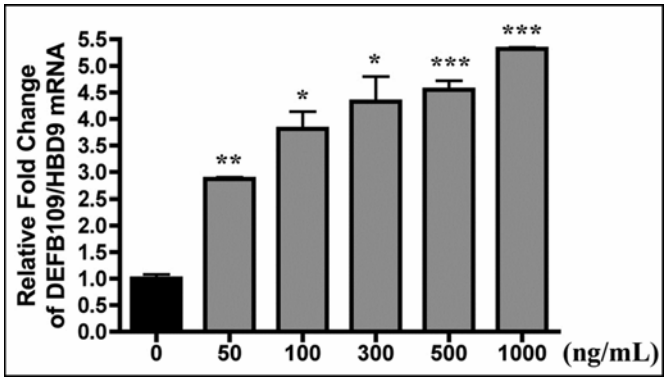

FIGURE 1

$(Palmitoyloxy)_3$-cysteinyl-serine-$(lysine)_4$ (Pam3CSK4) induces human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA) expression in dose-dependent manner. Cells were treated with different concentrations of Pam3CSK4 for 1 hour. HBD9 mRNA levels were analyzed by qPCR in treated cells against untreated control. Statistical analysis was performed using one-way ANOVA and Bonferroni posthoc test (**P*<.05; ***P*<.001; ****P*<.0001). Data represent means ± SEM of triplicate samples repeated three times.

DEXAMETHASONE (DEX) ATTENUATES TLR2-INDUCED HBD9 PROTEIN EXPRESSION

To elucidate the role of TLR2 in HBD9 protein expression, we performed immunofluorescence analysis using polyclonal antibody against HBD9 following stimulation of HCECs without or with Pam3CSK4 for 24 hours. As shown in Figure 2, protein staining of HBD9 was increased in treated cells (b) compared to untreated control (a). Moreover, to understand the effect of Dex on TLR2-induced HBD9 protein expression, cells were incubated with Dex before or after treatment with Pam3CSK4. Notably, in presence of Dex alone, a very modest increase in HBD9 protein staining was observed (c). However, introduction of Dex prior to Pam3CSK4 treatment led to reduction in HBD9 protein expression (d) compared to treatment with Pam3CSK4 alone (b).

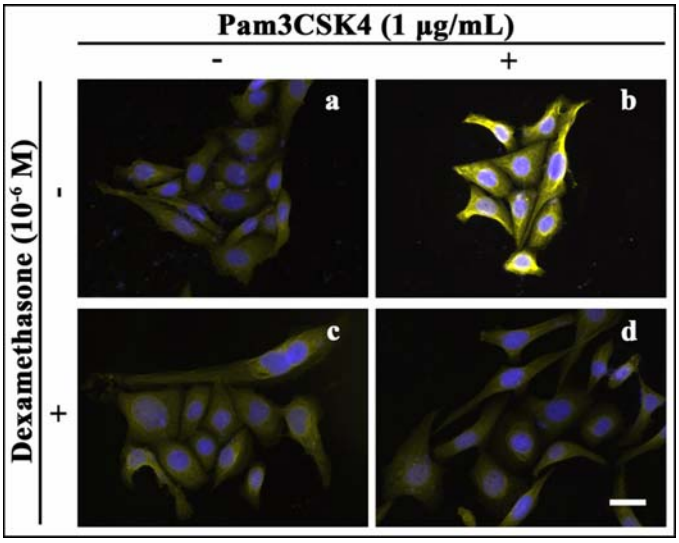

FIGURE 2

Dexamethasone (Dex) and (Palmitoyloxy)$_3$-cysteinyl-seryl-(lysine)$_4$ (Pam3CSK4) modulate human β-defensin 9 (HBD9) protein expression in human corneal epithelial cells. Cells were incubated in the absence (-) or presence (+) of Dex prior to treatment without (-) or with (+) Pam3CSK4. Panel a, untreated control; b, Pam3CSK4 alone showing increased expression; c, Dex alone showing modest increase; and d, Dex + Pam3CSK4 showing reduced expression compared to panel b, where cells were treated with Pam3CSK4 alone (magnification ×400, bar = 50 µM; blue = nuclei stained with 4′, 6-diamidino-2-phenylindole; yellow = immunofluorescent [fluorescein] staining of protein of interest). Data are representative of three independent experiments.

DEXAMETHASONE REDUCES TLR2-INDUCED HBD9 MRNA EXPRESSION

To investigate the effect of Dex on TLR2-induced HBD9 mRNA expression, we treated HCECs with different concentrations of Dex (10^{-9} M to 10^{-6} M, ie, 1 nM to 1000 nM) for 2 hours prior to stimulation with Pam3CSK4 (1 µg/mL) for 1 hour. Interestingly, Dex was shown to reduce TLR2-induced HBD9 mRNA levels in a concentration-dependent manner (Figure 3). In the presence of 10^{-6} M Dex alone (bar with dots), we noted a significant up-regulation of HBD9 mRNA expression in HCECs. Notably, the level of HBD9 mRNA achieved following incubation with Dex (10^{-6} M) in the presence (bar with dark ash grey colour) or absence (bar with dots) of Pam3CSK4 was similar.

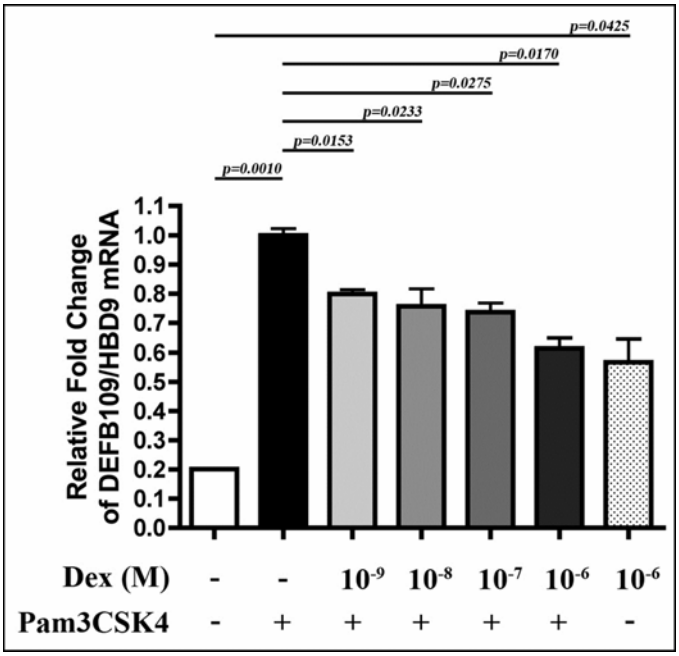

FIGURE 3

Dexamethasone (Dex) attenuates (Palmitoyloxy)$_3$-cysteinyl-seryl-(lysine)$_4$ (Pam3CSK4)–induced human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA) expression in dose-dependent manner. Cells were treated with different concentrations of Dex prior to Pam3CSK4 treatment. HBD9 mRNA levels were analyzed by qPCR in treated cells against Pam3CSK4 alone. Statistical analysis was performed using the Student *t* test. Significance was set at $P<.05$. Data represent means ± SEM of triplicate samples repeated two times.

TAK1 PLAYS A KEY ROLE IN HBD9 EXPRESSION IN HCECS

TAK1 is a protein kinase that controls several cell functions, which include regulation of transcription and apoptosis. It acts as a mediator in the transduction of signals initiated by TGF-β and morphogenetic protein. A complex of TAK1 is essential for the activation of NF-κB.

To understand the role of TAK-1 in TLR2-induced HBD9 expression, HCECs were incubated with preoptimized TAK-1 silencing RNA (siRNA [small interfering RNA], 1 nM) and negative control siRNA (10 nM) for 24 hours prior to treatment with Pam3CSK4 (1 µg/mL) for 1 hour. As shown in Figure 4, silencing TAK-1 in HCEC resulted in down-regulation of Pam3CSK4-induced HBD9 mRNA levels compared to negative siRNA pretreated cells. Similarly, to investigate the role of TAK-1 in HBD9 protein expression following TLR2 activation, cells were treated with negative siRNA or TAK-1 siRNA for 24 hours prior to stimulation with Pam3CSK4 (1 µg/mL; 24 hours). Treatment with TAK-1 siRNA (Figure 5B) resulted in reduced expression of HBD9 protein

compared to negative control siRNA-treated cells (Figure 5A). Thus, this suggests a key role of TAK-1 in up-regulation of HBD9 mRNA and protein upon TLR2 activation.

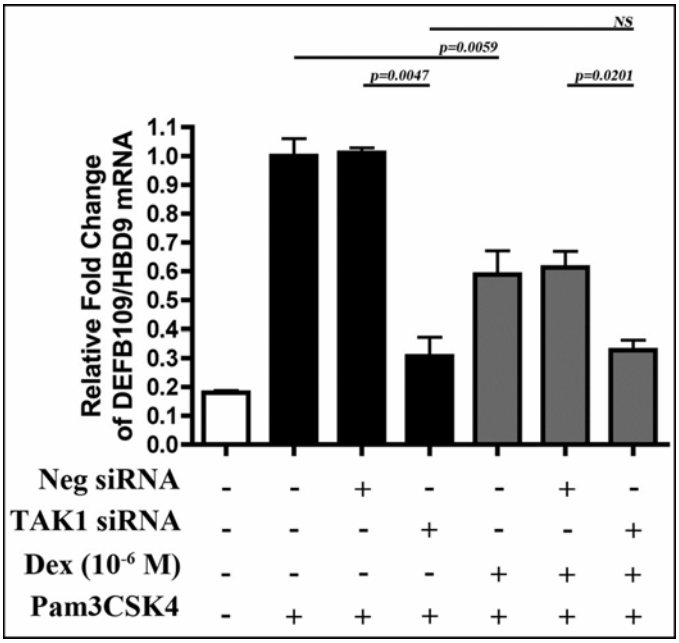

FIGURE 4

Transforming growth factor-β–activated kinase 1 (TAK-1) plays a central role in toll-like receptor 2 (TLR2)–induced human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA) expression in human corneal epithelial cells. White bar indicates untreated control. Black bars indicate HBD9 mRNA levels in cells incubated with negative control siRNA or TAK-1 siRNA prior to treatment with Pam3CSK4. Grey bars indicate HBD9 mRNA levels resulted from negative control siRNA or TAK-1 siRNA-treated cells in presence of Dex prior to Pam3CSK4 incubation. Statistical significance was measured between negative siRNA-treated cells and those with TAK-1 siRNA either in presence or absence of Dex. Data represent means ± SEM of triplicate samples repeated two times.

To elucidate further the effect of Dex on TAK-1-dependent TLR2-induced HBD9 mRNA expression, we incubated the siRNA (TAK-1 or negative control) pretreated (for 24 hours) cells with Dex for 2 hours prior to treatment with Pam3CSK4 (1 hour; 1 μg/mL). As shown in grey bars (Figure 4), treatment with Dex in the absence or presence of negative siRNA has resulted in attenuation of TLR2-induced HBD9 mRNA levels. Similarly, incubation of HCECs with Dex (2 hours) after treatment with TAK-1 siRNA (Figure 5D) and prior to stimulation with Pam3CSK4 for 24 hours has resulted in reduced expression of HBD9 protein compared to cells treated in similar fashion but in the presence of negative siRNA (Figure 5C). HBD9 mRNA and protein levels in cells incubated with Pam3CSK4 alone or with Dex + Pam3CSK4 combination after TAK-1 siRNA treatment were similar. This suggests that Dex has no direct effect on TAK-1–mediated inhibition of TLR2-induced HBD9 expression in HCECs.

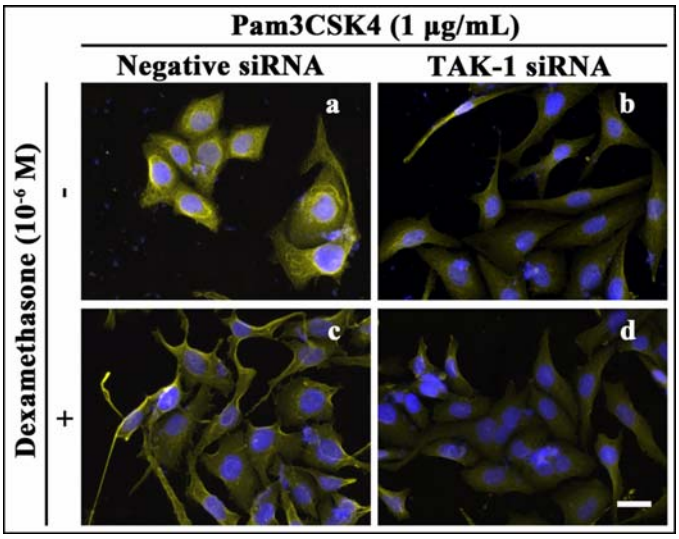

FIGURE 5

Transforming growth factor-β–activated kinase 1 (TAK-1) plays a central role in toll-like receptor 2 (TLR2)–induced human β-defensin 9 (HBD9) protein expression in human corneal epithelial cells. Cells were incubated in the presence of negative control siRNA (10 nM) or TAK-1 siRNA (1 nM) without (-) or with (+) Dex prior to treatment with Pam3CSK4. Panel a, negative (control) siRNA + Pam3CSK4 showing increased expression; b, TAK-1 siRNA + Pam3CSK4 showing reduced expression; c, negative (control) siRNA + Dex + Pam3CSK4 showing increased expression; and d, TAK-1 siRNA + Dex + Pam3CSK4 showing reduced expression. (magnification ×400, bar = 50 μM; blue = nuclei stained with 4′, 6-diamidino-2-phenylindole; yellow = immunofluorescent [fluorescein] staining of protein of interest). Data represent means ± SEM of triplicate samples repeated two times.

MAPKS AND NF-κB ARE INVOLVED IN TLR2-INDUCED HBD9 EXPRESSION

Mitogen-activated protein kinases are specific to eukaryotes and regulate cellular activity in response to a variety of stimuli, including mitogens and proinflammatory cytokines. They also regulate cell proliferation and apoptosis. To examine whether MAPKs (specifically, extracellular signal-regulated kinase [ERK], c-JUN NH2-terminal kinase [JNK], and p38 kinases) and NF-κB are involved in TLR2-mediated expression of HBD9, cells were incubated with inhibitors of p38 (50 μM SB203580), JNK (50 μM SP600125), ERK (50 μM PD98059), and NF-κB pathway (NAI and SC514; 10 μM each) for 30 minutes before treatment with Pam3CSK4 (1 μg/mL; 1 hour). As shown in Figure 6, blocking of MAPKs and NF-κB signalling pathways with specific inhibitors resulted in reduced expression of HBD9 mRNA compared to cells treated with Pam3CSK4 alone.

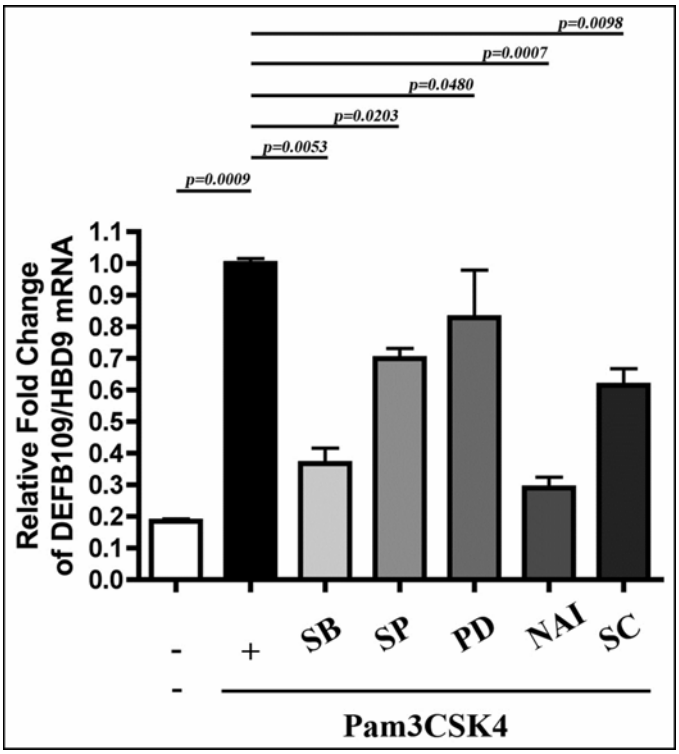

FIGURE 6

Nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) are involved in toll-like receptor 2 (TLR2)–induced increased expression of human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA). White bar indicates untreated control. Black bar indicates HBD9 mRNA levels in cells incubated with Pam3CSK4 alone. Treatments with inhibitors of p38 (SB203580), JNK (SP600125), ERK (PD98059), and NF-κB (NAI and SC514) prior to incubation with Pam3CSK4 are indicated in different shades of grey. Statistical significance was measured between Pam3CSK4 alone and those with inhibitors + Pam3CSK4. Data represent means ± SEM of triplicate samples repeated three times.

We then investigated the effect of MAPKs and NF-κB pathway inhibitors on the induction of expression of HBD9 protein by TLR2. As shown in Figure 7, HCECs treated with inhibitors of MAPKs and NF-κB (for 30 minutes) prior to stimulation with Pam3CSK4 (1 μg/mL) for 24 hours demonstrated a reduced staining pattern of HBD9 protein compared to cells treated with Pam3CSK4 alone. Interestingly, of all the MAPKs, inhibition of p38 pathway showed the most significant effect on HBD9 mRNA expression (Figure 6 SB) and protein expression (Figure 7B) in response to TLR2 activation. Blocking NF-κB pathway with NAI resulted in significant reduction of HBD9 mRNA (Figure 6 NAI) and protein expression (Figure 7F) with levels achieved similar to that after blocking p38 pathway. However, SC154 showed a modest effect on TLR2-induced HBD9 mRNA expression (Figure 6 SC) and protein (Figure 7E) expression, matching the levels achieved with JNK inhibitor. These results thus indicate an essential involvement of MAPKs and NF-κB pathway in induction of HBD9 transcript and protein by TLR2.

ROLE OF NF-κB1/P105 IN HBD9 EXPRESSION

NF-κB is a versatile transcription factor that is present in most cell types. It is involved in signal transduction and exists as homodimers or heterodimers. NF-κB1/p105 subunit is one such dimer. Complexes of NF-κB are present in the cytosol in an inactive state. Upon phosphorylation, active NF-κB is released, which translocates to the nucleus to contribute to signal transduction.

To investigate whether the NF-κB family member, NF-κB1/p105, has any role in TLR2-induced HBD9 expression, HCECs were incubated with NF-κB1/p105 siRNA or negative control siRNA for 24 hours before treatment with Pam3CSK4 (1 μg/mL) for 1 hour. Notably, cells pretreated with NF-κB1/p105 siRNA demonstrated significantly low levels of HBD9 mRNA compared to those treated with negative siRNA (Figure 8).

For HBD9 protein analysis, HCECs were treated with the above-mentioned siRNAs (for 24 hours) prior to stimulation with Pam3CSK4 for 24 hours. As shown in Figure 9, similar to HBD9 mRNA response, protein expression of HBD9 was also reduced in NF-κB1 siRNA-treated cells (d) compared to negative control siRNA-treated HCECs (c). This result suggests that NF-κB1/p105 is also involved in TLR2-induced HBD9 mRNA expression and protein formation.

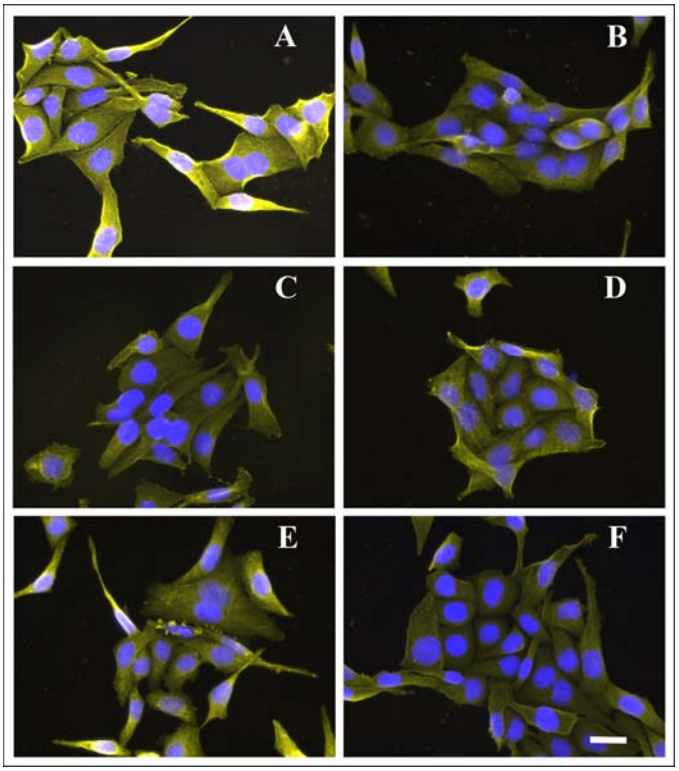

FIGURE 7

Nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) are involved in toll-like receptor 2 (TLR2)–induced increased expression of human β-defending 9 (HBD9) protein. Human corneal epithelial cells were treated with inhibitors of p38 (SB203580), JNK (SP600125), ERK (PD98059), and NF-κB (SC514 and NAI) prior to incubation with Pam3CSK4. Panel A, vehicle + Pam3CSK4. Panel B, SB + Pam3CSK4 showing the most reduction in expression of HBD9. Panel C, SP + Pam3CSK4. Panel D, PD + Pam3CSK4. Panel E, SC + Pam3CSK4. Panel F, NAI + Pam3CSK4 (magnification ×400, bar = 50 μM; blue = nuclei stained with 4′, 6-diamidino-2-phenylindole; yellow = immunofluorescent [fluorescein] staining of protein of interest). Data are representative of three independent experiments.

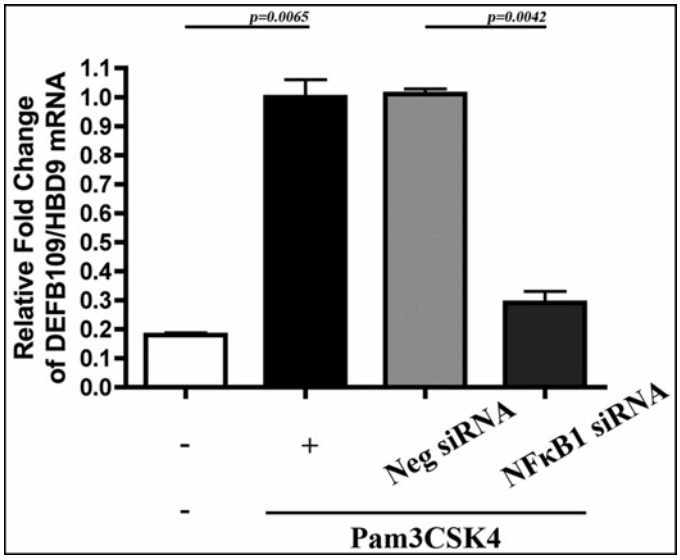

FIGURE 8

Nuclear factor-κB1/p105, a family member of nuclear factor-κB, also plays a role in toll-like receptor 2 (TLR2)–induced increased expression of human β-defencing 9 (HBD9) messenger ribonucleic acid (mRNA) expression in human corneal epithelial cells. White bar indicates untreated control. Black bar indicates HBD9 mRNA levels in cells treated with Pam3CSK4 alone. Grey bars indicate HBD9 mRNA levels resulting from negative control siRNA (10 nM) or NF-κB1/p105 siRNA (1 nM)–treated cells in presence of Pam3CSK4. Statistical significance was measured using the Student *t* test. Data represent means ± SEM of triplicate samples repeated three times.

C-JUN AND ATF2 ARE IMPORTANT REGULATORS OF HBD9 EXPRESSION IN RESPONSE TO TLR2 ACTIVATION

c-Jun N-terminal kinase is a member of the MAPK family that regulates a range of biological processes. Activating transcription factor 2 (ATF2) is a protein transcription factor. To examine the role of c-JUN and ATF2 in HBD9 expression following TLR2 activation, HCEC cells were treated with c-JUN siRNA or ATF2 siRNA for 24 hours followed by treatment with Pam3CSK4 (1 μg/mL; 1 hour). As shown in Figure 10, a significant down-regulation of HBD9 mRNA was noted in c-JUN siRNA-treated cells compared to those incubated with negative siRNA. Notably, HBD9 mRNA levels after c-JUN inhibition matched to the levels found in untreated control (white bar).

Likewise, ATF2 silencing prior to TLR2 activation with Pam3CSK4 also demonstrated decreased levels of HBD9 mRNA compared to negative siRNA-treated cells (Figure 11, black bars).

Furthermore, to elucidate the involvement of c-JUN and ATF2 in TLR2-mediated HBD9 protein expression, HCECs were treated with c-JUN siRNA or ATF-2 siRNA for 24 hours followed by stimulation with Pam3CSK4 (1 μg/mL, 24 hours). As shown in Figure 12, blocking c-JUN (b) or ATF-2 (c) in HCECs prior to TLR2 activation led to reduced staining of HBD9 protein compared to negative siRNA-treated cells (a). This indicates a role of both c-JUN and ATF-2 in induction of HBD9 in response to TLR2 activation in HCECs.

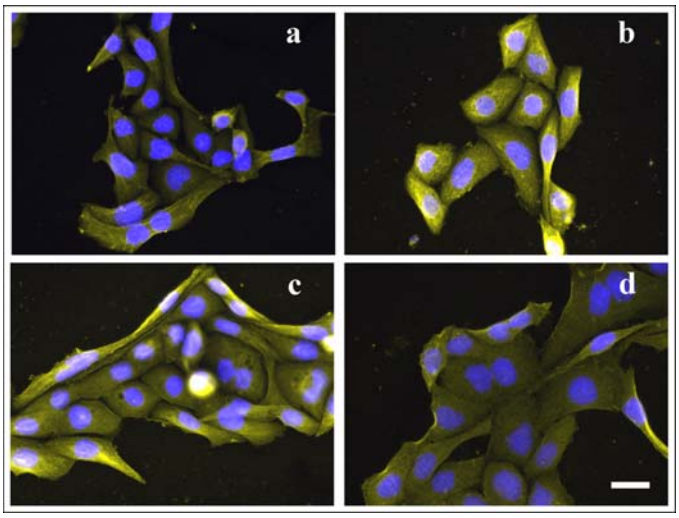

FIGURE 9

Nuclear factor-κB1/p105 also plays a role in toll-like receptor 2 (TLR2)–induced HBD9 protein expression in human corneal epithelial cells. Cells were incubated in the presence of negative control siRNA (10 nM) or NF-κB1/p105 siRNA (1 nM) prior to treatment with Pam3CSK4. Panel a, untreated control; b, Pam3CSK4 alone; c, Negative control siRNA + Pam3CSK4; and d, NF-κB1/p105 siRNA + Pam3CSK4, showing reduction in HBD9 expression. (magnification ×400, scale bar = 50 μM; blue = nuclei stained with 4′, 6-diamidino-2-phenylindole; yellow = immunofluorescent [fluorescein] staining of protein of interest). Data are representative of three independent experiments.

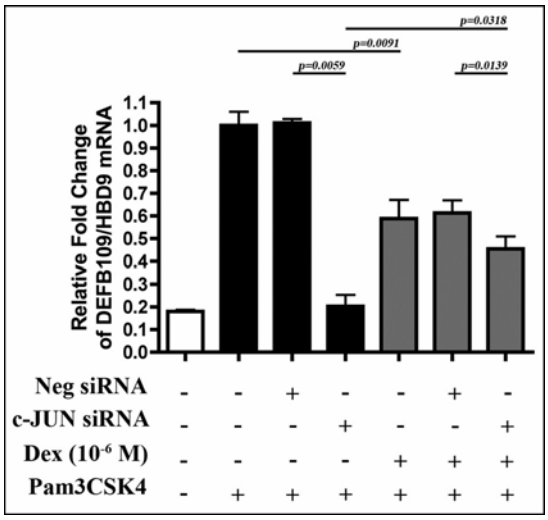

FIGURE 10

The transcription factor c-JUN is an essential regulator of toll-like receptor 2 (TLR2)–induced human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA) expression in human corneal epithelial cells. White bar indicates untreated control. Black bars indicate HBD9 mRNA levels in cells incubated with negative control siRNA (10 nM) or c-JUN siRNA (1 nM) prior to treatment with Pam3CSK4 (1 μg/mL). Grey bars indicate HBD9 mRNA levels resulting from negative control siRNA or c-JUN siRNA-treated cells in presence of Dex prior to Pam3CSK4 incubation. Statistical significance was measured between Negative siRNA-treated cells and those with c-JUN siRNA either in presence or absence of Dex. Data represent means ± SEM of triplicate samples repeated three times.

DEXAMETHASONE INDUCES HBD9 EXPRESSION IN C-JUN BUT NOT ATF2 SIRNA-TREATED HCECS

To analyze the effect of Dex on TLR2/transcription factors axis–mediated HBD9 mRNA expression, c-JUN siRNA or ATF2 siRNA pretreated HCECs were incubated with Dex for 2 hours before treatment with Pam3CSK4 (1 hour for mRNA analysis or 24 hours for protein analysis). HBD9 mRNA levels in HCECs treated with Dex + Pam3CSK4 in presence of c-JUN siRNA (Figure 10, grey bars) or ATF2 siRNA (Figure 11, grey bars) were found to be significantly reduced compared to those treated in similar fashion but in presence of negative siRNA. Notably, protein staining of HBD9 was shown to be lowered after silencing ATF2 (Figure 12 lower-right) but remained unchanged after silencing c-JUN (Figure 12E) in Dex + Pam3CSK4-treated HCECs compared to those treated in an identical manner but in presence of negative siRNA (Figure 12D). Interestingly, further analysis of results has revealed that HBD9 mRNA levels in c-JUN siRNA pretreated cells incubated with Dex + Pam3CSK4 (Figure 10, grey bar) were found to be significantly increased compared to those treated in similar fashion but in presence of Pam3CSK4 alone (Figure 10, black bar). Similarly, blocking c-JUN prior to Dex + Pam3CSK4 treatment (Figure 12E) has resulted in increased staining of HBD9 protein compared to those preincubated with c-JUN siRNA in an identical fashion but in presence of Pam3CSK4 alone (Figure 12b). However, no such difference in either HBD9 mRNA (Figure 11) or protein (Figure 12, C and F) levels has been noted in ATF2 siRNA-treated cells in presence or absence of Dex with Pam3CSK4. Therefore, this suggests a transactivation effect of Dex in TLR2-induced HBD9 expression in c-JUN-independent manner.

ROLE OF MKP-1 IN HBD9 EXPRESSION IN HCECS

Mitogen-activated protein kinase phosphatase 1 acts as a negative regulator of TLR signalling pathways, and mainly dephosphorylates the activated MAPKs.[157,158] To test whether MKP-1 has any role in TLR2-mediated HBD-9 expression, HCECs were incubated with MKP-1 siRNA or negative siRNA for 24 hours before treatment with Pam3CSK4 (1 μg/mL) for 1 hour. Silencing MKP-1 in HCECs resulted in further increase in TLR2-induced HBD9 mRNA levels compared to cells treated with negative siRNA (Figure 13, black bars).

Next, we investigated the effect of MKP-1 silencing on TLR2-activated HBD9 protein levels. As shown in Figure 14, HBD9 protein staining was increased following MKP-1 siRNA inhibition (Figure 14B) compared to HCECs pretreated with negative control siRNA (Figure 14A) prior to stimulation with Pam3CSK4 (24 hours). This indicates a negative role of MKP-1 in TLR2-induced HBD9 mRNA and protein expression.

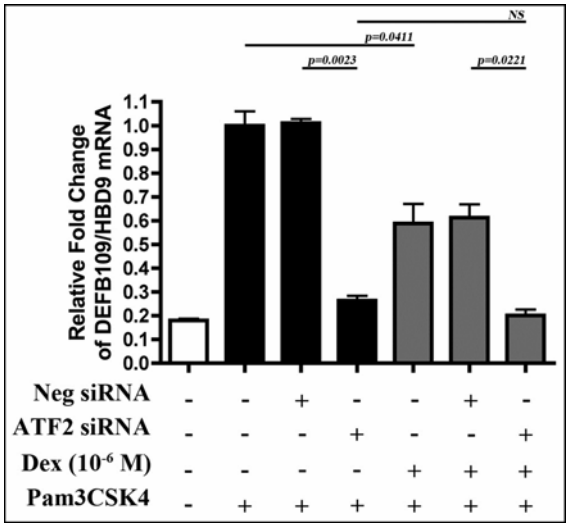

FIGURE 11

Activating transcription factor 2 (ATF2) is required for toll-like receptor 2 (TLR2)–induced human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA) expression in human corneal epithelial cells.White bar indicates untreated control. Black bars indicate HBD9 mRNA levels in cells incubated with negative control siRNA (10 nM) or ATF2 siRNA (1 nM) prior to treatment with Pam3CSK4 (1 μg/mL). Grey bars indicate HBD9 mRNA levels resulting from negative control siRNA or ATF2 siRNA-treated cells in presence of Dex prior to Pam3CSK4 incubation. Statistical significance was measured between negative siRNA-treated cells and those with ATF2 siRNA either in presence or absence of Dex. Data represent means ± SEM of triplicate samples repeated three times.

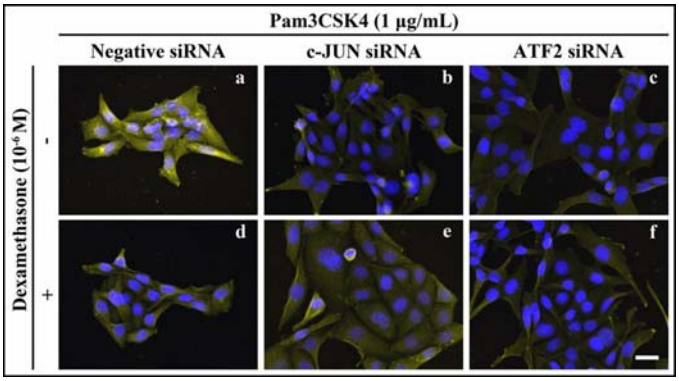

FIGURE 12

The transcription factor c-JUN and activating transcription factor 2 (ATF2) are important regulators of toll-like receptor 2 (TLR2)–induced human β-defensin 9 (HBD9) protein expression in human corneal epithelial cells. Cells were incubated in the presence of negative control siRNA (10 nM), c-JUN siRNA (1 nM), or ATF-2 siRNA (1 nM) without (-) or with (+) Dex prior to treatment with Pam3CSK4. Panel a, negative control siRNA + Pam3CSK4 showing increased expression of HBD9; b, c-JUN siRNA + Pam3CSK4 showing reduced expression; c, ATF-2 siRNA + Pam3CSK4 showing reduced expression; d, negative control siRNA + Dex + Pam3CSK4; e, c-JUN siRNA + Dex + Pam3CSK4; and f, ATF-2 siRNA + Dex + Pam3CSK4 (magnification ×400, scale bar = 50 μM; blue = nuclei stained with 4′, 6-diamidino-2-phenylindole; yellow = immunofluorescent [fluorescein] staining of protein of interest). Data are representative of three independent experiments.

DEXAMETHASONE INHIBITS TLR2-INDUCED HBD9 EXPRESSION VIA MKP-1 IN HCECS

Numerous studies have demonstrated the involvement of MKP1 in Dex-mediated inhibition of TLR and IL-1R-induced cytokine or AMP production.[159,160] To examine whether MKP1 is involved in the inhibitory effect of Dex on HBD9 expression mediated by TLR2, HCECs were incubated with MKP1 siRNA or negative siRNA for 24 hours before treatment with Dex for 2 hours and subsequently with Pam3CSK4 (1 hour for mRNA analysis or 24 hours for protein analysis). HBD9 mRNA levels were significantly enhanced after MKP-1 silencing in presence of Dex + Pam3CSK4 compared to those after negative siRNA pretreatment (Figure 13, grey bars). Notably, mRNA levels of HBD9 after MKP1 silencing prior to Dex + Pam3CSK4 treatment (grey bar) matched the HBD9 levels from cells after Pam3CSK4 treatment alone, in presence or absence of negative siRNA (black bar).

Likewise, as shown in Figure 14, MKP-1 silencing prior to Dex + Pam3CSK4 treatment has resulted in increased HBD9 protein staining in HCECs compared to those treated in similar fashion but in absence of Dex. Similar to HBD9 mRNA levels, protein staining of HBD9 in these cells matched the negative siRNA-treated HCECs in presence of Pam3CSK4 alone. Therefore, this indicates an essential role of MKP-1 in inhibitory effect of Dex on TLR2-induced HBD9 mRNA and protein expression in HCECs.

HBD9 AND OTHER AMP GENE EXPRESSION IN HCEC ON CHALLENGE WITH BACTERIA

P aeruginosa

AMP gene expression in HCECs stimulated with *P aeruginosa* showed a statistically significant up-regulation in 5 of the 8 AMPs targeted in this study (Figure 15). Only HBD9 and LEAP1 showed early and significant decreases in gene expression. Indeed, HBD9 was significantly down-regulated at 1 and 3 hours (Figure 15C), whereas LEAP1 showed statistically significant decrease at all time-points (Figure 15E). Gene expression of LEAP2 was variably increased (Figure 15F). Expression of HBD1, HBD2, HBD3, RNase-7, and LL-37 was significantly up-regulated with LL-37 reaching 10-fold increase at 6 hours (Figure 15G).

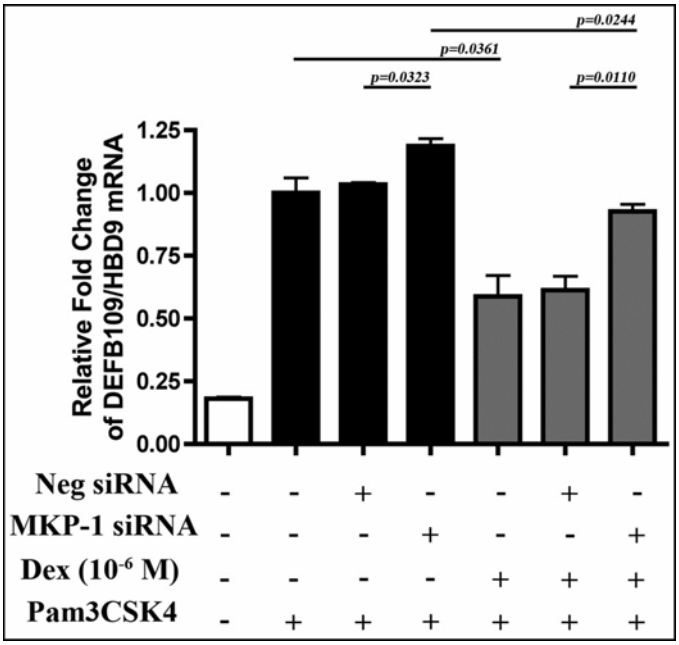

FIGURE 13

Role of mitogen-activated protein kinase phosphatase 1 (MKP-1) in human β-defensin 9 (HBD9) messenger ribonucleic acid (mRNA) expression. White bar indicates untreated control. Black bars indicate HBD9 mRNA levels in cells incubated with negative control siRNA (10 nM) or MKP-1 siRNA (1 nM) prior to treatment with Pam3CSK4 (1 μg/mL). Grey bars indicate HBD9 mRNA levels attained from negative control siRNA or MKP-1 siRNA-treated cells in presence of Dex prior to Pam3CSK4 incubation. Statistical significance was measured between negative siRNA-treated cells and those with MKP-1 siRNA either in presence or absence of Dex. Data represent means ± SEM of triplicate samples repeated three times.

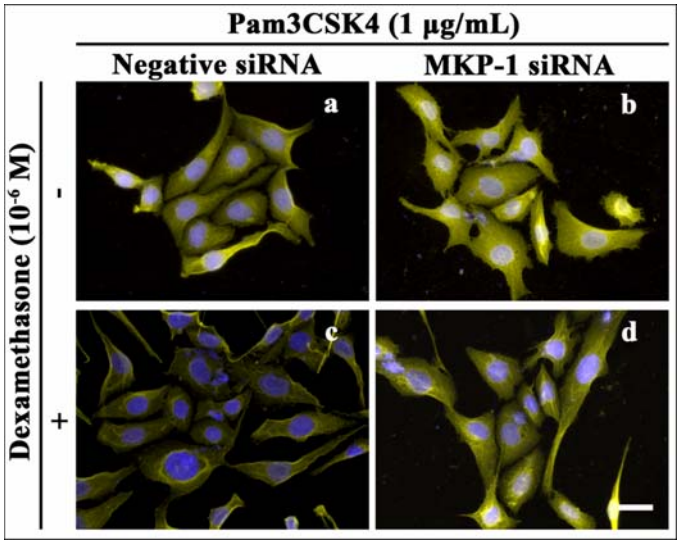

FIGURE 14

Role of mitogen-activated protein kinase phosphatase 1 (MKP-1) in human β-defensin 9 (HBD9) protein expression. Cells were incubated in the presence of negative control siRNA (10 nM) or MKP-1 siRNA (1 nM) without (-) or with (+) Dex prior to treatment with Pam3CSK4. Panel a, negative control siRNA + Pam3CSK4; b, MKP-1 siRNA + Pam3CSK4 showing increased expression of HBD9; c, negative control siRNA + Dex + Pam3CSK4; and d, MKP-1 siRNA + Dex + Pam3CSK4 (magnification ×400, scale bar = 50 μM; blue = nuclei stained with 4′, 6-diamidino-2-phenylindole; yellow = immunofluorescent [fluorescein] staining of protein of interest). Data are representative of three independent experiments.

S aureus

Each of the studied AMPs showed an overall up-regulation (Figure 16), but only LL37, LEAP2, and RNase-7 reached statistical significance at 1, 6, and 6 hours, respectively (Figures 16E, 16G, and 16H). Although it was not statistically significant, only the gene expression of HBD9 showed an immediate increase at 1 hour, followed by a gradual decline thereafter.

For all samples, qPCR did not detect a product when reverse transcriptase was not used during cDNA synthesis, thus excluding primer binding to genomic DNA and confirming the specific amplification of only cDNA (negative control). Similarly, no products were generated during negative control qPCR reactions of cDNA obtained from lysates of *P aeruginosa* or *S aureus* maintained in culture for 24 hours in the absence of HCECs.

DISCUSSION

In an earlier study on HBD9, contrary to our experience with other AMPs, we had demonstrated low levels of HBD9 mRNA expression at the human ocular surface during microbial infection.[76] This interesting observation led us to further investigate the potential inducers of HBD9, which we reported in a subsequent study where we had elucidated an important role of TLR2 in the induction of HBD9 mRNA using the immortalized HCEC model.[77] This encouraged us to explore and determine the signalling pathways involved in TLR2-mediated HBD9 expression.

We were able to confirm our previous observation that TLR2 is indeed the receptor that initiates the cascade inducing expression of HBD9 mRNA and its translation to the protein product in the HCEC model. TLR2 has previously been reported to induce expression of cytokines and β-defensins in a variety of cell types, including tracheobronchial epithelium,[161] intestinal epithelium,[150] skin keratinocytes,[162] and corneal epithelium.[149] Here, we have demonstrated for the first time an increased expression of both HBD9

mRNA and protein by TLR2.

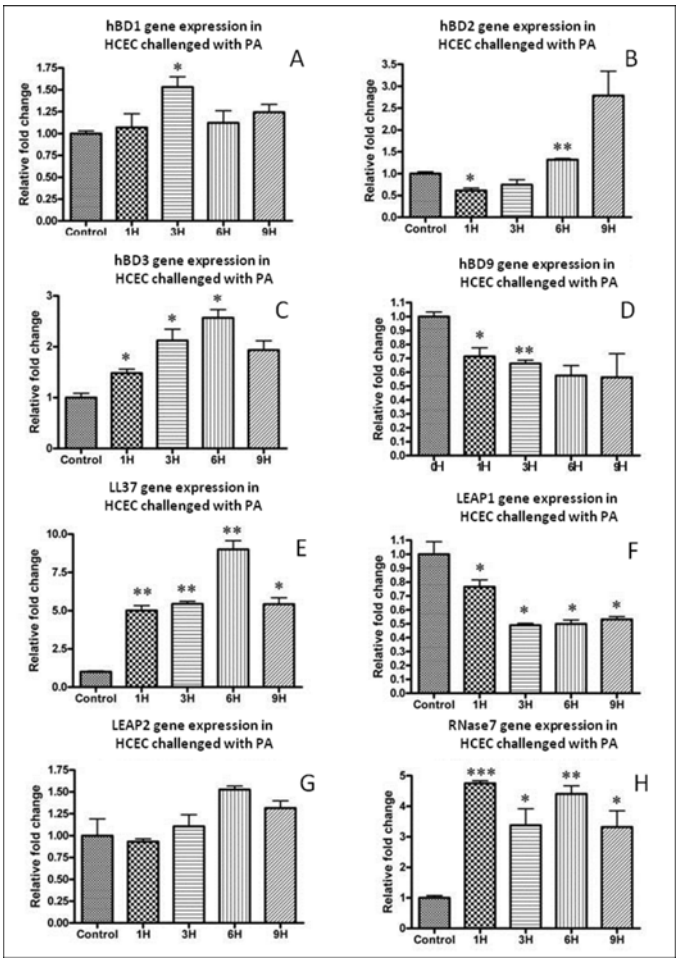

FIGURE 15

Antimicrobial peptide gene profile in human corneal epithelial cells (HCECs) challenged with *Pseudomonas aeruginosa*. hBD, human β-defensin; LL37, human cathelicidin; LEAP, liver-expressed antimicrobial peptide; PA, *Pseudomonas aureuginosa*; RNase7, ribonuclease 7. The x-axis shows time points in hours (**P*<.05, ***P*<.01, *** *P*<.001). All tests were done in triplicate with cells at the same passage number.

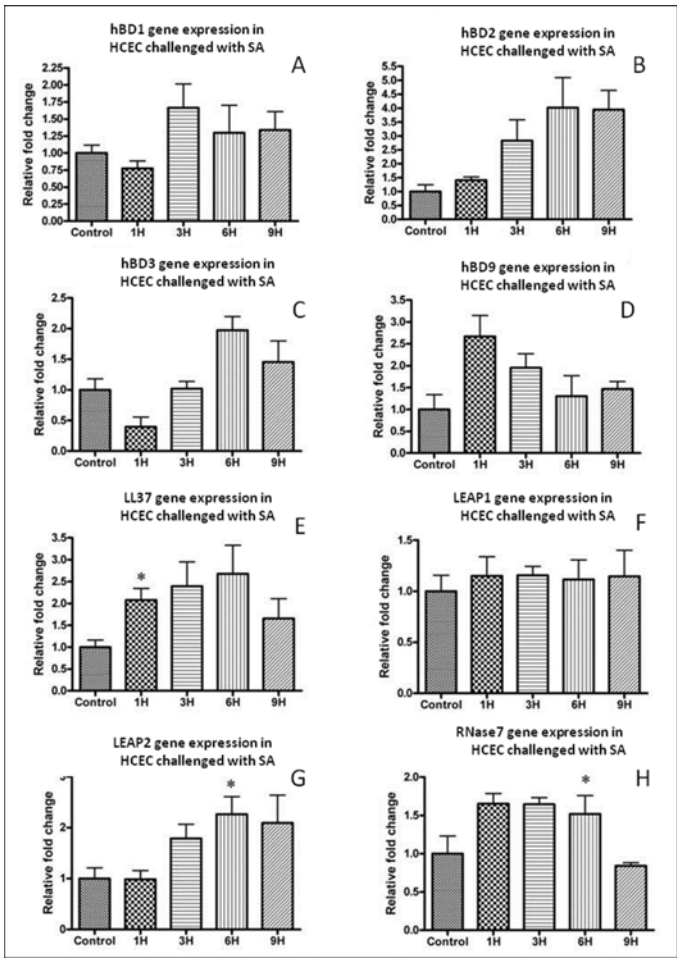

FIGURE 16

Antimicrobial peptide gene profile in human corneal epithelial cells (HCECs) challenged with *Staphylococcus aureus*. hBD, human β-defensin; LL37, human cathelicidin; LEAP, liver-expressed antimicrobial peptide; RNase7, ribonuclease 7; SA, *Staphylococcus aureus*. The x-axis shows time points in hours (**P*<.05, ** *P*<.01, ****P*<.001). All tests were done in triplicate with cells at the same passage number.

The study reveals that TAK-1, MAPKs, NF-κB, c-JUN, ATF2, and MKP-1 are involved in TLR2 related induction of HBD9. Use of both gene-specific siRNAs and pharmacologic inhibitors provided the evidence implicating the above key players in the signalling pathway of HBD9. We further demonstrated that dexamethasone could partially diminish the TLR2-mediated induction of HBD9 expression. This inhibitory effect of dexamethasone is specifically related to the interaction of dexamethasone with MKP-1. This may be one of the reasons that could explain diminished host defense and exacerbation of infection related to use of steroids during active keratitis. c-Jun was shown to play a specific role in dexamethasone effect, as in the absence of c-JUN, dexamethasone showed a modest induction of HBD9 in a TLR2-dependent manner.

Dexamethasone, a glucocorticoid, is widely used clinically due to its anti-inflammatory and immunosuppressive properties. It renders anti-inflammatory activity by transrepressing NF-κB-mediated proinflammatory gene expression[163] or by inducing the expression of MAPK phosphatase 1 (MKP-1), which effectively down-regulates MAPKs signalling pathway.[164] In addition to its transcriptional inhibition effect, dexamethasone has also been involved in transactivation of several genes, including *IκB*,[165] *MKP-1*,[166] and *HBD2*.[167] In this study, we have demonstrated both transactivating and transrepressing effects of dexamethasone on HBD9 expression. In presence of dexamethasone alone, HBD9 mRNA and protein levels were increased in HCECs. Using similar cell line model, Terai and colleagues[167] have demonstrated that the dexamethasone increases HBD2 but not HBD1 mRNA expression. However, it is still unclear how β-defensins are up-regulated in response to dexamethasone. Therefore, a thorough mechanistic study

needs to be carried out to further elucidate the transactivating abilities of dexamethasone on AMPs.

Several studies have provided the evidence on immunosuppressive effect of dexamethasone on TLR and IL-1β-mediated response. Jang and colleagues[160] have demonstrated that dexamethasone attenuates IL-1β-induced HBD2 expression in A549 cells. Similarly, McDermott's group[64] have also showed the down-regulation of HBD2 expression in response to dexamethasone in IL-1β-treated CECs. Recently, Winder and coworkers[159] have demonstrated that dexamethasone reduces TLR2/Pam3CSK4-induced HBD2 expression; however, a partial reversal of dexamethasone-negative activity on HBD2 occurred in the presence of cytokines. Consistent with these findings, we found that dexamethasone significantly reduces TLR2-induced HBD9 mRNA and protein expression in HCECs.

TAK-1 has been reported to play a key role in induction of innate and adaptive immune responses to a variety of stimuli.[168] Moreover, activation of TLRs, NLRs, IL-1R, and TNFR leads to phosphorylation of TAK-1, which in turn directly activates MAPK and NF-κB signalling pathways to induce host defense proteins.[168-172] In this study, we found that silencing of TAK-1 using gene-specific TAK-1 siRNA completely diminishes TLR2-induced HBD9 expression, suggesting a central role of TAK-1 in induction of HBD9 in response to TLR2 activation. A recent study[173] has shown that dexamethasone deactivates an upstream kinase molecule, TBK1, and subsequently IRF3 phosphorylation mediated by TLR3 and TLR4. However, there are no studies reporting the effect of dexamethasone on TAK1, an upstream kinase protein of TLR2 signalling pathway. In this study, we have shown that dexamethasone does not have any effect on TAK1-mediated HBD9 induction by TLR2 in HCECs. To this end, the role of TAK-1 in ocular surface immunity has not hitherto been determined using either in vivo or in vitro models. This is another area that requires further investigation.

NF-κB and MAPKs have widely been reported to modulate β-defensin expression in response to variety of stimuli on different cell surfaces. Wehkamp and colleagues[174] have demonstrated that activation of NF-κB and MAPKs in response to *P aeruginosa* and the cytokine IL-1β is essential for HBD2 induction in keratinocytes. In intestinal epithelial cells, induction of HBD2 expression has been shown to occur via both NF-κB and MAPKs in response to LPS and PGN, respectively.[150] At the ocular surface, HBD2 has been reported to increase in a NF-κB,- p38,- JNK-dependent manner, but not ERK-dependent manner, by IL-1β,[64] *S aureus,* and Pam3CSK4.[149] Therefore, given that AMPs are expressed via NF-κB or MAPKs, here, we studied the involvement of these in TLR2-mediated increased expression of HBD9 by blocking NF-κB, p38, JNK, and ERK pathways with specific inhibitors. Unlike HBD2 and RNase-7 expression at the ocular surface, our results indicate that both mRNA and protein expression of HBD9 is modulated by NF-κB and MAPKs (p38, JNK, and ERK) signalling pathways.

TLRs induce innate immune responses via activation of NF-κB or AP-1 (c-JUN/ATF2 or c-JUN/c-Fos) transcription factors in a variety of cell types. In response to LPS, TLR4 has been reported to induce the expression of IL-23p90[175] and cyclo-oxygenase-2 (COX-2)[176] in macrophages via activation of c-JUN and ATF2. Numerous studies have demonstrated an increased expression of HBD2 in intestinal,[150] tracheobronchial,[177] and airway epithelial cells[178] in response to activation of transcription factors NF-κB and AP-1 via TLR2 or TLR4. In addition to NF-κB (p65-p50 heterodimer), HBD2 is also induced via p50-p50 homodimer in response to LPS in mononuclear phagocytes.[179] In the present study, we investigated the involvement of NFkB1/p105 (precursor of p50 molecule), c-JUN, and ATF2 transcription factors in HBD9 expression by TLR2. Small interfering RNA (siRNA)-mediated silencing of these transcription factors resulted in attenuation of TLR2-induced HBD9 mRNA and protein expression. These findings suggest that HBD9 expression in response to TLR2 activation is mainly mediated via NF-κB, c-JUN, and ATF2. Studies are under way to analyze the binding site of these transcription factors on the promoter region of the HBD9 gene.

It is evident from numerous studies that c-JUN interacts with glucocorticoid receptor (GR) and inhibits GR-mediated transactivation or transrepression activities of dexamethasone.[180-182] However, it is still unclear whether such interaction between c-JUN and GR has any effect on cross talk between TLR and dexamethasone. Unexpectedly, we have shown that RNAi knockdown of c-JUN prior to dexamethasone treatment has resulted in TLR2-dependent induction of HBD9 mRNA and protein expression. In contrast, no such effect was demonstrated after silencing ATF2. Based on these results, we propose that c-JUN plays an important role in cross talk between TLR2 and dexamethasone in modulation of HBD9 expression. However, to understand this better, it would be necessary to study the effect of c-JUN silencing on GR transcription and vice versa and also the interaction of GR with other transcription factors in relation to the cross talk between dexamethasone and TLRs.

MAPK phosphatase has been reported to regulate TLR-mediated innate immune responses in both in vivo and in vitro models.[157,158,183] However, the involvement of MKP-1 in regulation of AMP expression is less understood. In this study, we investigated the role of MKP-1 in TLR2-mediated HBD9 expression by silencing MKP-1 with gene-specific siRNA. Cells deficient in MKP-1 prior to TLR2 activation have demonstrated a further enhancement of TLR2-induced HBD9 mRNA and protein expression. Thus, our results demonstrate that MKP-1 plays a crucial role in negative feedback control of TLR2-mediated HBD9 expression. Further studies elucidating the exact mechanism by which MKP-1 negatively regulates HBD9 expression are clearly needed. The MKP family consists of 11 members, each capable of inducing a negative effect at different levels and against different targets.[184] Of all, MKP-1, -2, -3, and -5 have been shown to play a negative feedback role in TLR-induced innate and adaptive immunity.[185-187] It would therefore be important to study MKP-2, -3, and -5 in addition to MKP-1 in regulation of TLR-mediated AMP expression.

Immunosuppressive action of dexamethasone on infectious or inflammatory stimuli–induced NF-κB and MAPKs-mediated responses are exerted through an increased production of MKP-1.[160,188-191] Here, we have demonstrated that silencing of MKP-1 in the presence of dexamethasone prior to TLR2 activation resulted in a reversal of the dexamethasone inhibitory effect on TLR2-induced HBD9 mRNA and protein expression. Consistent with previous studies with other defensins, we report that MKP-1 is crucial for dexamethasone-mediated inhibition of TLR2-induced HBD9 expression.

We also studied the expression of HBD9 following exposure of HCEC to *P aeruginosa* and *S aureus.* This was compared to an array of other AMPs. All the AMPs studied were shown to be constitutively expressed by unchallenged HCEC. Constitutive expression of HBD1-3,[6,74] HBD9,[76,77] LL37,[6,74] LEAP 1 and 2,[6] and RNase-7[79] in ocular surface cells has been previously reported. In addition, the inducible nature of the HBD3 has also been reported by McIntosh and colleagues,[6] who detected a greater expression of HBD3 in ocular surface cultures taken from infected corneas than in those from healthy corneas. Similarly, HBD2 expression was also found to be inducible by proinflammatory cytokines and bacterial products.[56,64,65,70,192]

In this study we noted an up-regulation of 6 of the 8 and all eight of the studied AMPs following HCEC challenge with *P aeruginosa* and *S aureus*, respectively. This increase was statistically significant in 5 AMPs for *P aeruginosa*–treated cells and in 4 AMPs for *S aureus*–treated cells. LL37 and HBD2 showed the highest levels of up-regulation in both groups, with ninefold and fourfold increases, respectively. HBD9 expression also showed some variation with the two pathogens tested. With *P aeruginosa* HBD9 was down-regulated at all the time points studied, but with *S aureus* an early up-regulation was observed, which gradually and steadily decreased over time. This is consistent with previous reports, including our own,[76,152] of reduced expression of HBD9 in samples of ocular surface cells taken from patients with infectious keratitis and dry eye[76] and in gingival keratinocytes.[152] In another study we reported an initial increase in HBD9 mRNA levels followed by a significant down-regulation in response to PAMPs and inflammatory cytokines stimulation.[77]

The expression profiles of the AMPs that were up-regulated were largely similar but not identical in cells treated with the two microbes, suggesting that the cell response is in part specific for the invading organism. This may reflect the different spectrum of PAMPs present on gram-negative and gram-positive bacteria.[193]

In this study we have presented evidence to indicate that TLR2 induces HBD9 mRNA and protein expression in a time- and dose-dependent manner. TAK-1 plays a central role in HBD9 induction by TLR2. An involvement of c-JUN and ATF2 transcription factors in HBD9 expression in response to TLR2 activation is also indicated. We report that dexamethasone reduces TLR2-mediated up-regulation of HBD9 mRNA and protein levels in MKP-1 dependent or c-JUN-independent manner. These pathway-specific molecules can be exploited to modulate the response of HBD9 during microbial infection. The diverse and vast array of AMPs expressed at the ocular surface points to the significant host defense advantage nature has conferred to the preservation of sight. The variable expression of different AMPs to specific pathogens would suggest similar but subtly different pathways invoked by the pathogens, probably related to their PAMPs and different TLRs and other receptors they bind to.

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Author Contributions: *Design and conduct of the study* (H.D., A.O., A.H., I.M.); *Collection, management, analysis, and interpretation of the data* (H.D., A.O., A.H., I.M.); *Preparation* (H.D.), *review* (H.D., A.O., A.H., I.M.), *and approval of the manuscript* (H.D.).

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REFERENCES

1. Department of Pathology and Microbiology, University of Nebraska Medical Center. The antimicrobial peptide database. Available at: http://aps.unmc.edu/AP/main.php. Accessed December 23, 2013.
2. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415(6870):389-395.
3. Silva NC, Sarmento B, Pintado M. The importance of antimicrobial peptides and their potential for therapeutic use in ophthalmology. *Int J Antimicrob Agents* 2013;41(1):5-10.
4. Wiesner J, Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* 2010;1(5):440-464.
5. Hazlett L, Wu M. Defensins in innate immunity. *Cell Tissue Res* 2011;343(1):175-188.
6. McIntosh RS, Cade JE, Al-Abed M, et al. The spectrum of antimicrobial peptide expression at the ocular surface. *Invest Ophthalmol Vis Sci* 2005;46(4):1379-1385.
7. Huang LC, Jean D, Proske RJ, Reins RY, McDermott AM. Ocular surface expression and in vitro activity of antimicrobial peptides. *Curr Eye Res* 2007;32(7-8):595-609.
8. Stack D, Neville C, Doyle S. Nonribosomal peptide synthesis in *Aspergillus fumigatus* and other fungi. *Microbiology* 2007;153(Pt 5):1297-1306.
9. Schwarzer D, Mootz HD, Linne U, Marahiel MA. Regeneration of misprimed nonribosomal peptide synthetases by type II thioesterases. *Proc Natl Acad Sci U S A* 2002;99(22):14083-14088.
10. Stein T, Vater J, Kruft V, et al. The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic templates. *J Biol Chem* 1996;271(26):15428-15435.
11. Vizioli J, Salzet M. Antimicrobial peptides from animals: focus on invertebrates. *Trends Pharmacol Sci* 2002;23(11):494-496.
12. Ganz T, Selsted ME, Szklarek D, et al. Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* 1985;76(4):1427-1435.

13. Nguyen TX, Cole AM, Lehrer RI. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* 2003;24(11):1647-1654.
14. McDermott AM. The role of antimicrobial peptides at the ocular surface. *Ophthalmic Res* 2009;41(2):60-75.
15. Uchio E, Inoue H, Kadonosono K. Anti-adenoviral effects of human cationic antimicrobial protein-18/LL-37, an antimicrobial peptide, by quantitative polymerase chain reaction. *Korean J Ophthalmol* 2013;27(3):199-203.
16. Yount NY, Wang MS, Yuan J, Banaiee N, Ouellette AJ, Selsted ME. Rat neutrophil defensins. Precursor structures and expression during neutrophilic myelopoiesis. *J Immunol* 1995;155(9):4476-4484.
17. Ganz T, Liu L, Valore EV, Oren A. Posttranslational processing and targeting of transgenic human defensin in murine granulocyte, macrophage, fibroblast, and pituitary adenoma cell lines. *Blood* 1993;82(2):641-650.
18. Ganz T, Lehrer RI. Defensins. *Curr Opin Immunol* 1994;6(4):584-589.
19. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. *J Biol Chem* 1992;267(32):23216-23225.
20. Cunliffe RN, Rose FR, Keyte J, Abberley L, Chan WC, Mahida YR. Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut* 2001;48(2):176-185.
21. Ouellette AJ, Satchell DP, Hsieh MM, Hagen SJ, Selsted ME. Characterization of luminal paneth cell alpha-defensins in mouse small intestine. Attenuated antimicrobial activities of peptides with truncated amino termini. *J Biol Chem* 2000;275(43):33969-33973.
22. Quayle AJ, Porter EM, Nussbaum AA, et al. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol* 1998;152(5):1247-1258.
23. Wu M, Peng A, Sun M, et al. TREM-1 amplifies corneal inflammation after *Pseudomonas aeruginosa* infection by modulating Toll-like receptor signaling and Th1/Th2-type immune responses. *Infect Immun* 2011;79(7):2709-2716.
24. Augustin DK, Heimer SR, Tam C, et al. Role of defensins in corneal epithelial barrier function against *Pseudomonas aeruginosa* traversal. *Infect Immun* 2011;79(2):595-605.
25. Meloni M, De Servi B, Marasco D, Del Prete S. Molecular mechanism of ocular surface damage: application to an in vitro dry eye model on human corneal epithelium. *Mol Vis* 2011;17:113-126.
26. Jager K, Nielitz A, Garreis F, Sel S, Nave H, Paulsen FP. In vitro evidence of involvement of the epithelial y+ transporter in beta-defensin production on the ocular surface. *Ann Anat* 2011;193(6):479-485.
27. Berger EA, McClellan SA, Vistisen KS, Hazlett LD. HIF-1alpha is essential for effective PMN bacterial killing, antimicrobial peptide production and apoptosis in *Pseudomonas aeruginosa* keratitis. *PLoS Pathog* 2013;9(7):e1003457.
28. Soehnlein O, Lindbom L. Neutrophil-derived azurocidin alarms the immune system. *J Leukoc Biol* 2009;85(3):344-351.
29. Park PJ, Antoine TE, Farooq AV, Valyi-Nagy T, Shukla D. An investigative peptide-acyclovir combination to control herpes simplex virus type 1 ocular infection. *Invest Ophthalmol Vis Sci* 2013;54(9):6373-6381.
30. Tang YQ, Yuan J, Osapay G, et al. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* 1999;286(5439):498-502.
31. De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol Lett* 2005;27(18):1337-1347.
32. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun* 1995;63(4):1291-1297.
33. Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect Dis* 2001;1(3):156-164.
34. Giacometti A, Cirioni O, Barchiesi F, Caselli F, Scalise G. In-vitro activity of polycationic peptides against *Cryptosporidium parvum*, *Pneumocystis carinii* and yeast clinical isolates. *J Antimicrob Chemother* 1999;44(3):403-406.
35. Chalekson CP, Neumeister MW, Jaynes J. Improvement in burn wound infection and survival with antimicrobial peptide D2A21 (Demegel). *Plast Reconstr Surg* 2002;109(4):1338-1343.
36. Bastian A, Schafer H. Human alpha-defensin 1 (HNP-1) inhibits adenoviral infection in vitro. *Regul Pept* 2001;101(1-3):157-161.
37. Harmsen MC, Swart PJ, de Bethune MP, et al. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. *J Infect Dis* 1995;172(2):380-388.
38. Sinha S, Cheshenko N, Lehrer RI, Herold BC. NP-1, a rabbit alpha-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. *Antimicrob Agents Chemother* 2003;47(2):494-500.
39. Gropp R, Frye M, Wagner TO, Bargon J. Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. *Hum Gene Ther* 1999;10(6):957-964.
40. Buck CB, Day PM, Thompson CD, et al. Human alpha-defensins block papillomavirus infection. *Proc Natl Acad Sci U S A* 2006;103(5):1516-1521.
41. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 1996;86(6):973-983.
42. Wilson CL, Ouellette AJ, Satchell DP, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 1999;286(5437):113-117.
43. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 1997;88(4):553-560.

44. Nagaoka I, Hirota S, Yomogida S, Ohwada A, Hirata M. Synergistic actions of antibacterial neutrophil defensins and cathelicidins. *Inflamm Res* 2000;49(2):73-79.
45. Midorikawa K, Ouhara K, Komatsuzawa H, et al. *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infect Immun* 2003;71(7):3730-739.
46. Maisetta G, Batoni G, Esin S, et al. In vitro bactericidal activity of human beta-defensin 3 against multidrug-resistant nosocomial strains. *Antimicrob Agents Chemother* 2006;50(2):806-809.
47. Niyonsaba F, Iwabuchi K, Someya A, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* 2002;106(1):20-26.
48. Scott MG, Yan H, Hancock RE. Biological properties of structurally related alpha-helical cationic antimicrobial peptides. *Infect Immun* 1999;67(4):2005-2009.
49. Marr AK, Gooderham WJ, Hancock RE. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr Opin Pharmacol* 2006;6(5):468-472.
50. Steinberg DA, Hurst MA, Fujii CA, et al. Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. *Antimicrob Agents Chemother* 1997;41(8):1738-1742.
51. Evans DJ, Fleiszig SM. Why does the healthy cornea resist *Pseudomonas aeruginosa* infection? *Am J Ophthalmol* 2013;155(6):961-970.e2.
52. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* 2003;55(1):27-55.
53. Stolzenberg ED, Anderson GM, Ackermann MR, Whitlock RH, Zasloff M. Epithelial antibiotic induced in states of disease. *Proc Natl Acad Sci U S A* 1997;94(16):8686-8690.
54. Haynes RJ, Tighe PJ, Dua HS. Innate defence of the eye by antimicrobial defensin peptides. *Lancet* 1998;352(9126):451-452.
55. Gottsch JD, Li Q, Ashraf MF, O'Brien TP, Stark WJ, Liu SH. Defensin gene expression in the cornea. *Curr Eye Res* 1998;17(11):1082-1086.
56. Haynes RJ, Tighe PJ, Dua HS. Antimicrobial defensin peptides of the human ocular surface. *Br J Ophthalmol* 1999;83(6):737-741.
57. Haynes RJ, McElveen JE, Dua HS, Tighe PJ, Liversidge J. Expression of human beta-defensins in intraocular tissues. *Invest Ophthalmol Vis Sci* 2000;41(10):3026-3031.
58. Lehmann OJ, Hussain IR, Watt PJ. Investigation of beta defensin gene expression in the ocular anterior segment by semiquantitative RT-PCR. *Br J Ophthalmol* 2000;84(5):523-526.
59. McDermott AM. Antimicrobial compounds in tears. *Exp Eye Res* 2013;117:53-61.
60. Li N, Wang N, Zheng J, et al. Characterization of human tear proteome using multiple proteomic analysis techniques. *J Proteome Res* 2005;4(6):2052-2061.
61. Ohashi Y, Dogru M, Tsubota K. Laboratory findings in tear fluid analysis. *Clin Chim Acta* 2006;369(1):17-28.
62. Zhou L, Huang LQ, Beuerman RW, et al. Proteomic analysis of human tears: defensin expression after ocular surface surgery. *J Proteome Res* 2004;3(3):410-416.
63. McNamara NA, Van R, Tuchin OS, Fleiszig SM. Ocular surface epithelia express mRNA for human beta defensin-2. *Exp Eye Res* 1999;69(5):483-490.
64. McDermott AM, Redfern RL, Zhang B, Pei Y, Huang L, Proske RJ. Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2003;44(5):1859-1865.
65. Narayanan S, Miller WL, McDermott AM. Expression of human beta-defensins in conjunctival epithelium: relevance to dry eye disease. *Invest Ophthalmol Vis Sci* 2003;44(9):3795-3801.
66. Kolar SS, McDermott AM. Role of host-defence peptides in eye diseases. *Cell Mol Life Sci* 2011;68(13):2201-2213.
67. Singh PK, Jia HP, Wiles K, et al. Production of beta-defensins by human airway epithelia. *Proc Natl Acad Sci U S A* 1998;95(25):14961-14966.
68. Liu L, Wang L, Jia HP, et al. Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 1998;222(2):237-244.
69. Shin JS, Kim CW, Kwon YS, Kim JC. Human beta-defensin 2 is induced by interleukin-1beta in the corneal epithelial cells. *Exp Mol Med* 2004;36(3):204-210.
70. McDermott AM, Redfern RL, Zhang B. Human beta-defensin 2 is up-regulated during re-epithelialization of the cornea. *Curr Eye Res* 2001;22(1):64-67.
71. Kawasaki S, Kawamoto S, Yokoi N, et al. Up-regulated gene expression in the conjunctival epithelium of patients with Sjogren's syndrome. *Exp Eye Res* 2003;77(1):17-26.
72. Otri AM, Mohammed I, Al-Aqaba MA, et al. Variable expression of human Beta defensins 3 and 9 at the human ocular surface in infectious keratitis. *Invest Ophthalmol Vis Sci* 2012;53(2):757-761.
73. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* 2001;276(8):5707-5713.
74. McDermott AM. Defensins and other antimicrobial peptides at the ocular surface. *Ocul Surf* 2004;2(4):229-247.
75. Garreis F, Schlorf T, Worlitzsch D, et al. Roles of human beta-defensins in innate immune defense at the ocular surface: arming and alarming corneal and conjunctival epithelial cells. *Histochem Cell Biol* 2010;134(1):59-73.

76. Abedin A, Mohammed I, Hopkinson A, Dua HS. A novel antimicrobial peptide on the ocular surface shows decreased expression in inflammation and infection. *Invest Ophthalmol Vis Sci* 2008;49(1):28-33.
77. Mohammed I, Suleman H, Otri AM, et al. Localization and gene expression of human beta-defensin 9 at the human ocular surface epithelium. *Invest Ophthalmol Vis Sci* 2010;51(9):4677-4682.
78. Garreis F, Gottschalt M, Schlorf T, et al. Expression and regulation of antimicrobial peptide psoriasin (S100A7) at the ocular surface and in the lacrimal apparatus. *Invest Ophthalmol Vis Sci* 2011;52(7):4914-4922.
79. Mohammed I, Yeung A, Abedin A, Hopkinson A, Dua HS. Signalling pathways involved in ribonuclease-7 expression. *Cell Mol Life Sci* 2011;68(11):1941-1952.
80. Rodriguez-Martinez S, Cancino-Diaz ME, Cancino-Diaz JC. Expression of CRAMP via PGN-TLR-2 and of alpha-defensin-3 via CpG-ODN-TLR-9 in corneal fibroblasts. *Br J Ophthalmol* 2006;90(3):378-382.
81. Diamond G, Kaiser V, Rhodes J, Russell JP, Bevins CL. Transcriptional regulation of beta-defensin gene expression in tracheal epithelial cells. *Infect Immun* 2000;68(1):113-119.
82. National Center for Biotechnology Information. Databases and tools. Available at: http://www.ncbi.nlm.nih.gov/guide/all/. Accessed December 23, 2013.
83. Sack RA, Nunes I, Beaton A, Morris C. Host-defense mechanism of the ocular surfaces. *Biosci Rep* 2001;21(4):463-480.
84. Huang LC, Petkova TD, Reins RY, Proske RJ, McDermott AM. Multifunctional roles of human cathelicidin (LL-37) at the ocular surface. *Invest Ophthalmol Vis Sci* 2006;47(6):2369-2380.
85. Campanelli D, Detmers PA, Nathan CF, Gabay JE. Azurocidin and a homologous serine protease from neutrophils. Differential antimicrobial and proteolytic properties. *J Clin Invest* 1990;85(3):904-915.
86. Ruan X, Chodosh J, Callegan MC, et al. Corneal expression of the inflammatory mediator CAP37. *Invest Ophthalmol Vis Sci* 2002;43(5):1414-1421.
87. Tang YQ, Yeaman MR, Selsted ME. Antimicrobial peptides from human platelets. *Infect Immun* 2002;70(12):6524-6533.
88. Huff T, Muller CS, Otto AM, Netzker R, Hannappel E. beta-Thymosins, small acidic peptides with multiple functions. *Int J Biochem Cell Biol* 2001;33(3):205-220.
89. Sosne G, Chan CC, Thai K, et al. Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. *Exp Eye Res* 2001;72(5):605-608.
90. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, Hazlett LD. Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. *Exp Eye Res* 2002;74(2):293-299.
91. Schittek B, Hipfel R, Sauer B, et al. Dermcidin: a novel human antibiotic peptide secreted by sweat glands. *Nat Immunol* 2001;2(12):1133-1137.
92. Rieg S, Garbe C, Sauer B, Kalbacher H, Schittek B. Dermcidin is constitutively produced by eccrine sweat glands and is not induced in epidermal cells under inflammatory skin conditions. *Br J Dermatol* 2004;151(3):534-539.
93. Kao CY, Huang F, Chen Y, et al. Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. *J Immunol* 2005;175(10):6676-6685.
94. Shirane J, Nakayama T, Nagakubo D, et al. Corneal epithelial cells and stromal keratocytes efficently produce CC chemokine-ligand 20 (CCL20) and attract cells expressing its receptor CCR6 in mouse herpetic stromal keratitis. *Curr Eye Res* 2004;28(5):297-306.
95. Puinhas A, Sampaio P, Castanheira EM, Real Oliveira ME, Lira M. Comparison of IgA, TNF-alpha and surface tension of the tear film in two different times of the day. *Cont Lens Anterior Eye* 2013;36(3):140-145.
96. Dua HS. Bacterial keratitis in the critically ill and comatose patient. *Lancet* 1998;351(9100):387-388.
97. Fung K, Morris C, Duncan M. Mass spectrometric techniques applied to the analysis of human tears: a focus on the peptide and protein constituents. *Adv Exp Med Biol* 2002;506(Pt A):601-605.
98. Stoeckelhuber M, Stoeckelhuber BM, Welsch U. Human glands of Moll: histochemical and ultrastructural characterization of the glands of Moll in the human eyelid. *J Invest Dermatol* 2003;121(1):28-36.
99. Berry M, Harris A, Lumb R, Powell K. Commensal ocular bacteria degrade mucins. *Br J Ophthalmol* 2002;86(12):1412-1416.
100. Holweg A, Schnare M, Gessner A. The bactericidal/permeability-increasing protein (BPI) in the innate defence of the lower airways. *Biochem Soc Trans* 2011;39(4):1045-1050.
101. Peuravuori H, Aho VV, Aho HJ, Collan Y, Saari KM. Bactericidal/permeability-increasing protein in lacrimal gland and in tears of healthy subjects. *Graefes Arch Clin Exp Ophthalmol* 2006;244(2):143-148.
102. Leitch EC, Willcox MD. Synergic antistaphylococcal properties of lactoferrin and lysozyme. *J Med Microbiol* 1998;47(9):837-842.
103. Ellison RT 3rd, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest* 1991;88(4):1080-1091.
104. Bals R. Epithelial antimicrobial peptides in host defense against infection. *Respir Res* 2000;1(3):141-150.
105. Singh PK, Tack BF, McCray PB Jr, Welsh MJ. Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. *Am J Physiol Lung Cell Mol Physiol* 2000;279(5):L799-805.
106. Yan H, Hancock RE. Synergistic interactions between mammalian antimicrobial defense peptides. *Antimicrob Agents Chemother* 2001;45(5):1558-1560.
107. Schutte BC, McCray PB Jr. [beta]-defensins in lung host defense. *Annu Rev Physiol* 2002;64:709-748.
108. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem* 1998;273(6):3718-3724.

109. Spencer LT, Paone G, Krein PM, Rouhani FN, Rivera-Nieves J, Brantly ML. Role of human neutrophil peptides in lung inflammation associated with alpha1-antitrypsin deficiency. *Am J Physiol Lung Cell Mol Physiol* 2004;286(3):L514-520.
110. Turner HC, Budak MT, Akinci MA, Wolosin JM. Comparative analysis of human conjunctival and corneal epithelial gene expression with oligonucleotide microarrays. *Invest Ophthalmol Vis Sci* 2007;48(5):2050-2061.
111. Wei Y, Pinhas A, Liu Y, Epstein S, Wang J, Asbell P. Isoforms of secretory group two phospholipase A (sPLA2) in mouse ocular surface epithelia and lacrimal glands. *Invest Ophthalmol Vis Sci* 2012;53(6):2845-2855.
112. Franken C, Meijer CJ, Dijkman JH. Tissue distribution of antileukoprotease and lysozyme in humans. *J Histochem Cytochem* 1989;37(4):493-498.
113. Sathe S, Sakata M, Beaton AR, Sack RA. Identification, origins and the diurnal role of the principal serine protease inhibitors in human tear fluid. *Curr Eye Res* 1998;17(4):348-362.
114. Sallenave JM. Secretory leukocyte protease inhibitor and elafin/trappin-2: versatile mucosal antimicrobials and regulators of immunity. *Am J Respir Cell Mol Biol* 2010;42(6):635-643.
115. Stahlman MT, Gray ME, Hull WM, Whitsett JA. Immunolocalization of surfactant protein-D (SP-D) in human fetal, newborn, and adult tissues. *J Histochem Cytochem* 2002;50(5):651-660.
116. Ni M, Evans DJ, Hawgood S, Anders EM, Sack RA, Fleiszig SM. Surfactant protein D is present in human tear fluid and the cornea and inhibits epithelial cell invasion by *Pseudomonas aeruginosa*. *Infect Immun* 2005;73(4):2147-2156.
117. Brauer L, Kindler C, Jager K, et al. Detection of surfactant proteins A and D in human tear fluid and the human lacrimal system. *Invest Ophthalmol Vis Sci* 2007;48(9):3945-3953.
118. You J, Fitzgerald A, Cozzi PJ, et al. Post-translation modification of proteins in tears. *Electrophoresis* 2010;31(11):1853-1861.
119. Ma P, Wang N, McKown RL, Raab RW, Laurie GW. Focus on molecules: lacritin. *Exp Eye Res* 2008;86(3):457-458.
120. McDermott AM, Rich D, Cullor J, et al. The in vitro activity of selected defensins against an isolate of *Pseudomonas* in the presence of human tears. *Br J Ophthalmol* 2006;90(5):609-611.
121. Travis SM, Anderson NN, Forsyth WR, et al. Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect Immun* 2000;68(5):2748-2755.
122. Panyutich AV, Hiemstra PS, van Wetering S, Ganz T. Human neutrophil defensin and serpins form complexes and inactivate each other. *Am J Respir Cell Mol Biol* 1995;12(3):351-357.
123. Paulsen FP, Pufe T, Schaudig U, et al. Detection of natural peptide antibiotics in human nasolacrimal ducts. *Invest Ophthalmol Vis Sci* 2001;42(10):2157-2163.
124. Paulsen FP, Pufe T, Schaudig U, et al. Protection of human efferent tear ducts by antimicrobial peptides. *Adv Exp Med Biol* 2002;506(Pt A):547-553.
125. Thakur A, Willcox MD. Chemotactic activity of tears and bacteria isolated during adverse responses. *Exp Eye Res* 1998;66(2):129-137.
126. Dartt DA. Control of mucin production by ocular surface epithelial cells. *Exp Eye Res* 2004;78(2):173-185.
127. Aarbiou J, Verhoosel RM, Van Wetering S, et al. Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. *Am J Respir Cell Mol Biol* 2004;30(2):193-201.
128. Dyer KD, Rosenberg HF. The RNase a superfamily: generation of diversity and innate host defense. *Mol Divers* 2006;10(4):585-597.
129. Carlsson G, Fasth A. Infantile genetic agranulocytosis, morbus Kostmann: presentation of six cases from the original "Kostmann family" and a review. *Acta Paediatr* 2001;90(7):757-764.
130. Putsep K, Carlsson G, Boman HG, Andersson M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* 2002;360(9340):1144-1149.
131. Ganz T, Metcalf JA, Gallin JI, Boxer LA, Lehrer RI. Microbicidal/cytotoxic proteins of neutrophils are deficient in two disorders: Chediak-Higashi syndrome and "specific" granule deficiency. *J Clin Invest* 1988;82(2):552-556.
132. Shiohara M, Gombart AF, Sekiguchi Y, et al. Phenotypic and functional alterations of peripheral blood monocytes in neutrophil-specific granule deficiency. *J Leukoc Biol* 2004;75(2):190-197.
133. Nomura I, Goleva E, Howell MD, et al. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 2003;171(6):3262-3269.
134. Frohm M, Agerberth B, Ahangari G, et al. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* 1997;272(24):15258-15263.
135. Harder J, Schroder JM. Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *J Leukoc Biol* 2005;77(4):476-486.
136. Harder J, Bartels J, Christophers E, Schroder JM. A peptide antibiotic from human skin. *Nature* 1997;387(6636):861.
137. Narayanan S, Redfern RL, Miller WL, Nichols KK, McDermott AM. Dry eye disease and microbial keratitis: Is there a connection? *Ocul Surf* 2013;11(2):75-92.
138. Brito BE, Zamora DO, Bonnah RA, Pan Y, Planck SR, Rosenbaum JT. Toll-like receptor 4 and CD14 expression in human ciliary body and TLR-4 in human iris endothelial cells. *Exp Eye Res* 2004;79(2):203-208.
139. Malmsten M, Kasetty G, Pasupuleti M, Alenfall J, Schmidtchen A. Highly selective end-tagged antimicrobial peptides derived from PRELP. *PLoS One* 2011;6(1):e16400.
140. Welling MM, Hiemstra PS, van den Barselaar MT, et al. Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. *J Clin Invest* 1998;102(8):1583-1590.

141. Bals R, Weiner DJ, Moscioni AD, Meegalla RL, Wilson JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun* 1999;67(11):6084-6089.
142. Huang GT, Zhang HB, Kim D, Liu L, Ganz T. A model for antimicrobial gene therapy: demonstration of human beta-defensin 2 antimicrobial activities in vivo. *Hum Gene Ther* 2002;13(17):2017-2025.
143. Hancock RE, Chapple DS. Peptide antibiotics. *Antimicrob Agents Chemother* 1999;43(6):1317-1323.
144. Sousa LB, Mannis MJ, Schwab IR, et al. The use of synthetic Cecropin (D5C) in disinfecting contact lens solutions. *CLAO J* 1996;22(2):114-117.
145. Schwab IR, Dries D, Cullor J, et al. Corneal storage medium preservation with defensins. *Cornea* 1992;11(5):370-375.
146. Mannis MJ. The use of antimicrobial peptides in ophthalmology: an experimental study in corneal preservation and the management of bacterial keratitis. *Trans Am Ophthalmol Soc* 2002;100:243-271.
147. Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J. Human beta-defensins. *Cell Mol Life Sci* 2006;63(11):1294-1313.
148. Bensch KW, Raida M, Magert HJ, Schulz-Knappe P, Forssmann WG. hBD-1: a novel beta-defensin from human plasma. *FEBS Lett* 1995;368(2):331-335.
149. Kumar A, Zhang J, Yu FS. Toll-like receptor 2-mediated expression of beta-defensin-2 in human corneal epithelial cells. *Microbes Infect* 2006;8(2):380-389.
150. Vora P, Youdim A, Thomas LS, et al. Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J Immunol* 2004;173(9):5398-5405.
151. Nagy I, Pivarcsi A, Koreck A, Szell M, Urban E, Kemeny L. Distinct strains of *Propionibacterium acnes* induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. *J Invest Dermatol* 2005;124(5):931-938.
152. Premratanachai P, Joly S, Johnson GK, McCray PB Jr, Jia HP, Guthmiller JM. Expression and regulation of novel human beta-defensins in gingival keratinocytes. *Oral Microbiol Immunol* 2004;19(2):111-117.
153. Mohammed I, Abedin A, Tsintzas K, et al. Increased expression of hepcidin and toll-like receptors 8 and 10 in viral keratitis. *Cornea* 2011;30(8):899-904.
154. Otri AM, Mohammed I, Abedin A, et al. Antimicrobial peptides expression by ocular surface cells in response to *Acanthamoeba castellanii*: an in vitro study. *Br J Ophthalmol* 2010;94(11):1523-1527.
155. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci* 1995;36(3):614-621.
156. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25(2):169-193.
157. Salojin KV, Owusu IB, Millerchip KA, Potter M, Platt KA, Oravecz T. Essential role of MAPK phosphatase-1 in the negative control of innate immune responses. *J Immunol* 2006;176(3):1899-1907.
158. Zhao Q, Wang X, Nelin LD, et al. MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. *J Exp Med* 2006;203(1):131-140.
159. Winder AA, Wohlford-Lenane C, Scheetz TE, et al. Differential effects of cytokines and corticosteroids on Toll-like receptor 2 expression and activity in human airway epithelia. *Respir Res* 2009;10:96.
160. Jang BC, Lim KJ, Suh MH, Park JG, Suh SI. Dexamethasone suppresses interleukin-1beta-induced human beta-defensin 2 mRNA expression: involvement of p38 MAPK, JNK, MKP-1, and NF-kappaB transcriptional factor in A549 cells. *FEMS Immunol Med Microbiol* 2007;51(1):171-184.
161. Hertz CJ, Wu Q, Porter EM, et al. Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol* 2003;171(12):6820-6826.
162. Sumikawa Y, Asada H, Hoshino K, et al. Induction of beta-defensin 3 in keratinocytes stimulated by bacterial lipopeptides through toll-like receptor 2. *Microbes Infect* 2006;8(6):1513-1521.
163. King EM, Holden NS, Gong W, Rider CF, Newton R. Inhibition of NF-kappaB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. *J Biol Chem* 2009;284(39):26803-26815.
164. Abraham SM, Lawrence T, Kleiman A, et al. Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *J Exp Med* 2006;203(8):1883-1889.
165. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 1995;270(5234):286-290.
166. Johansson-Haque K, Palanichamy E, Okret S. Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP. *J Mol Endocrinol* 2008;41(4):239-249.
167. Terai K, Sano Y, Kawasaki S, et al. Effects of dexamethasone and cyclosporin A on human beta-defensin in corneal epithelial cells. *Exp Eye Res* 2004;79(2):175-180.
168. Sato S, Sanjo H, Takeda K, et al. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol* 2005;6(11):1087-1095.
169. Chen CM, Gong Y, Zhang M, Chen JJ. Reciprocal cross-talk between Nod2 and TAK1 signaling pathways. *J Biol Chem* 2004;279(24):25876-25882.
170. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* 1999;398(6724):252-256.

171. Kim JY, Omori E, Matsumoto K, Nunez G, Ninomiya-Tsuji J. TAK1 is a central mediator of NOD2 signaling in epidermal cells. *J Biol Chem* 2008;283(1):137-144.
172. Schuman J, Chen Y, Podd A, et al. A critical role of TAK1 in B-cell receptor-mediated nuclear factor kappaB activation. *Blood* 2009;113(19):4566-4574.
173. McCoy CE, Carpenter S, Pålsson-McDermott EM, Gearing LJ, O'Neill LA. Glucocorticoids inhibit IRF3 phosphorylation in response to Toll-like receptor-3 and -4 by targetingTBK1 activation. *J Biol Chem* 2008;23;283(21):14277-14285.
174. Wehkamp K, Schwichtenberg L, Schroder JM, Harder J. *Pseudomonas aeruginosa*- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is controlled by NF-kappaB and AP-1. *J Invest Dermatol* 2006;126(1):121-127.
175. Liu W, Ouyang X, Yang J, et al. AP-1 activated by toll-like receptors regulates expression of IL-23 p19. *J Biol Chem* 2009;284(36):24006-24016.
176. Cho IJ, Kim SG. A novel mitogen-activated protein kinase phosphatase-1 and glucocorticoid receptor (GR) interacting protein-1-dependent combinatorial mechanism of gene transrepression by GR. *Mol Endocrinol* 2009;23(1):86-99.
177. Becker MN, Diamond G, Verghese MW, Randell SH. CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem* 2000;275(38):29731-29736.
178. Wang X, Zhang Z, Louboutin JP, Moser C, Weiner DJ, Wilson JM. Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. *FASEB J* 2003;17(12):1727-1729.
179. Tsutsumi-Ishii Y, Nagaoka I. NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation. *J Leukoc Biol* 2002;71(1):154-162.
180. Schule R, Rangarajan P, Kliewer S, et al. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* 1990;62(6):1217-1226.
181. Cabral AL, Hays AN, Housley PR, Brentani MM, Martins VR. Repression of glucocorticoid receptor gene transcription by c-Jun. *Mol Cell Endocrinol* 2001;175(1-2):67-79.
182. Maroder M, Farina AR, Vacca A, et al. Cell-specific bifunctional role of Jun oncogene family members on glucocorticoid receptor-dependent transcription. *Mol Endocrinol* 1993;7(4):570-584.
183. Chi H, Barry SP, Roth RJ, et al. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proc Natl Acad Sci U S A* 2006;103(7):2274-2279.
184. Lang R, Hammer M, Mages J. DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. *J Immunol* 2006;177(11):7497-7504.
185. Chu Y, Solski PA, Khosravi-Far R, Der CJ, Kelly K. The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *J Biol Chem* 1996;271(11):6497-6501.
186. Castelli M, Camps M, Gillieron C, et al. MAP kinase phosphatase 3 (MKP3) interacts with and is phosphorylated by protein kinase CK2alpha. *J Biol Chem* 2004;279(43):44731-44739.
187. Zhang Y, Blattman JN, Kennedy NJ, et al. Regulation of innate and adaptive immune responses by MAP kinase phosphatase 5. *Nature* 2004;430(7001):793-797.
188. Bhavsar PK, Sukkar MB, Khorasani N, Lee KY, Chung KF. Glucocorticoid suppression of CX3CL1 (fractalkine) by reduced gene promoter recruitment of NF-kappaB. *FASEB J* 2008;22(6):1807-1816.
189. Castro-Caldas M, Mendes AF, Carvalho AP, Duarte CB, Lopes MC. Dexamethasone prevents interleukin-1beta-induced nuclear factor-kappaB activation by upregulating IkappaB-alpha synthesis, in lymphoblastic cells. *Mediators Inflamm* 2003;12(1):37-46.
190. Bladh LG, Johansson-Haque K, Rafter I, Nilsson S, Okret S. Inhibition of extracellular signal-regulated kinase (ERK) signaling participates in repression of nuclear factor (NF)-kappaB activity by glucocorticoids. *Biochim Biophys Acta* 2009;1793(3):439-446.
191. Clark AR, Lasa M. Crosstalk between glucocorticoids and mitogen-activated protein kinase signalling pathways. *Curr Opin Pharmacol* 2003;3(4):404-411.
192. Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr Eye Res* 2005;30(7):505-515.
193. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* 2009;22(2):240-273.